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THE UNIVERSITY OF ALBERTA

PATTERN DUPLICATIONS IN A MUTANT OF *DROSOPHILA MELANOGASTER*

BY

JACK RICHARD GIRTON

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Pattern Duplications in a mutant of *Drosophila melanogaster* submitted by Jack Richard Girton in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

In this thesis the cell autonomous temperature sensitive lethal mutation *l(1)ts726* was used to induce pattern duplications of the mesothoracic leg of *Drosophila*. Two main studies were done on these duplications: (i) a detailed phenotypic analysis and (ii) a clonal analysis of the intermediate stages of development.

In the phenotypic analysis, 893 duplications were scored. In these duplications the morphological markers in the medial portion of the disc were often deficient and rarely duplicated, while the more lateral markers were rarely deficient and often duplicated. This pattern is similar to that seen in experiments involving the culturing of leg disc fragments. The deficiencies associated with these duplications often affected very few markers. These results were compared with the predictions of the polar coordinate model of French et al. (1976). Several possible explanations for the difference between the observed size of the deficiencies accompanying these duplications and the predictions of the model were considered.

Two clonal analyses of the intermediate stages of development were done, one using the *Minute* technique (Garcia-Bellido et al. 1973) to induce large clones. From the non-*Minute* clones, estimations were made of the number of cells present in the duplications at several times in development. These estimates were very similar to estimates made of normal leg discs during embryogenesis. The *Minute* clones were scored for location. Those clones induced by late irradiation were found to be restricted by a boundary identical to the anterior/posterior compartment boundary in normal legs. The results of these clonal analyses suggests that duplications are growing by a reiteration of normal development.

These results are discussed in the light of the two leading views of *Drosophila* development. One hypothesis, based on clonal analyses, suggests that cells become sequentially determined throughout development. The second, based on regeneration studies suggests that positional information systems specify each cell location and thus determine which structures it will form. The present results suggest that a compromise is possible between these views, that cells interpret their positional information via a series of sequential steps, and that regeneration occurs when cells "forget" certain previous decisions and reiterate portions of the sequence.

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INTRODUCTION

One aspect of development currently receiving a great deal of attention is the process whereby pattern is established (Wolpert 1969, 1971, Bryant 1971, Schubiger 1971, Postlethwait and Schneiderman 1973, 1974, Lawrence 1970, 1973, Russell 1974, Garcia-Bellido 1975, Postlethwait 1975, Bryant 1975, Wolpert et al. 1975, French 1976 a,b, French et al. 1976, Strub 1977 a,b, Russell et al. 1977). This question is a central one for multicellular organisms. Not only must each cell be instructed as to how it is to differentiate, but the individual cells must also be directed to differentiate in proper spatial arrays. The process whereby cells select such a single specific pathway of development has been termed "determination" (Hadorn 1965). Operationally, cells are assumed to pass their state of determination on to their offspring. Thus whenever the progeny of a single cell can be shown to be restricted in their developmental potential (eg., to forming only one of two structures), the original cell is said to have been determined.

It is believed that in *Drosophila* a sequence of determinative events occurs, with cells becoming progressively restricted throughout development. Some of this evidence comes from the analysis of genetic mosaics (see Stern 1968, Gehring and Nöthinger 1972, Postlethwait and Schneiderman 1974 and Hall et al. 1976 for review). One type of mosaic, the XX-XO gynandromorph, is produced by the loss of one X chromosome from a nucleus in a female embryo very early in development (Morgan 1914, Sturtevant 1929, Patterson and Stone 1938). In these gynandromorphs the patches of male (XO) tissue are observed to mark all visible structures in the adult at random, indicating that the nucleus in which the elimina-

tion occurred was not determined to form any particular adult structures. Studies of chromosome elimination at the third to fourth nuclear division, induced by the mutant *mit* (Gelbart 1971, 1974) provide a similar result. No restrictions are seen on the structures formed by the XO patches. Thus prior to the blastoderm stage, the nuclei appear to be not determined to form any particular adult structures.

In addition the determinative state of preblastoderm nuclei has been tested directly. Illmensee (1968, 1970, 1972, 1976) and Okada et al. (1974) removed individual nuclei from genetically marked embryos prior to the cellular blastoderm stage and transplanted them into unfertilized eggs or embryos at earlier stages of development. The descendents of these nuclei could form all larval and adult structures. This result also indicates that prior to cellular blastoderm, nuclei are not determined.

The earliest stage of development for which evidence for determination has been found is at cellular blastoderm, when the nuclei in the cortex are first incorporated into cells. Chan and Gehring (1971) observed that at this stage cells were restricted in their developmental potential. They divided genetically marked blastoderm stage embryos into anterior and posterior halves. Each half was dissociated into single cells and mixed with dissociated, wild type, whole embryos and cultured in the abdomen of an adult female. Under these conditions the cells proliferated. After several days in culture they were induced to undergo metamorphosis by injecting them into a late third instar larva. Cells from the anterior portion of the blastoderm produced head and thoracic structures while posterior cells produced abdomen and thoracic structures. These results indicate that cells at the blastoderm

stage are no longer able to form all of the adult structures, i.e., they have become determined for anterior or posterior structures. The observation that both fragments produced thoracic structures may be the result of variations in the location of the bissection from case to case or may indicate that some cells in both portions had the potential to form thoracic structures.

In a more direct examination of the determinative state of blastoderm cells Illmensee (1976) transplanted single, genetically marked blastoderm cells into host embryos which were also at the cellular blastoderm stage. He observed that the descendents of these cells formed structures characteristic of the original location of the transplanted cell, irrespective of their location in the host. This agrees with the results of Chan and Gehring in suggesting that cells at the cellular blastoderm stage had become determined, but the results published so far do not answer the question as to how specific is the state of determination at the blastoderm stage.

More detailed studies on this point have been made using the technique of mitotic recombination to produce somatic mosaics (Stern 1936). Mosaics can be induced at any time during development by exposing *Drosophila* which are heterozygous for marker mutations to ionizing radiation (Becker 1957). Mitotic recombination can result in two daughter cells homozygous for portions of the parental chromosomes. By using appropriate genetic markers the progeny of one or both cells can be detected as a patch of marked cuticle in the adult. Thus a state of determination can be inferred when clones induced at a given stage cease to include both of the two arbitrarily chosen morphological markers. The technique has the disadvantage that clones induced later in develop-

ment may be too small to mark both structures, even though the original cell had the potential to form both.

To increase the effectiveness of this technique at the later stages of development several investigators have made use of a class of mutants known as *Minutes* (Stern 1936, Kaplan 1953, Lindsley and Grell 1968). These are recessive lethals, some of which show a dominant cell autonomous decrease in cell division rates (Stern and Tikunaga 1971). When somatic cell clones which are *Minute*$^{+}$ are induced in a heterozygous *Minute* background, the cells of the clone grow faster than the background cells and produce large clones (Morata and Ripoll 1975).

Several investigators have applied the *Minute* technique to the study of the state of determination of cells at about the cellular blastoderm stage (3-4 hours) (Steiner 1976, Wieschaus and Gehring 1976a). Clones induced at this time did not cross from segment to segment or from the anterior into the posterior within a segment. They did however, mark structures formed by more than one imaginal disc, eg., Steiner (1976) observed clones which marked both the wing and the mesothoracic leg. The observed clonal restrictions imply that at the time of initiation of these clones the cells had become determined to form a specific portion of a specific segment, but, were not yet determined to form a specific imaginal disc. Clones induced later, at 10 hours after egg laying marked only structures formed by a single imaginal disc (Steiner 1976, Wieschaus and Gehring 1976a, Lawrence and Morata 1977). These results suggest that the cells potential to form adult structures is progressively restricted by a sequence of events in early development. The results of the cell culture and transplantation experiments (Chan and Gehring 1971, Illmensee 1976) suggests that these restrictions may be determinative events.

A series of investigators have detected similar clonal restrictions at later stages in the growth of imaginal discs (Becker 1957, Garcia-Bellido and Merriam 1971, Garcia-Bellido et al. 1973). The areas defined by these restrictions have been termed "compartments". In the wing disc compartmentalization events occur in a regular temporal sequence in which existing compartments are progressively subdivided (see Garcia-Bellido 1975 for the location of the boundaries in the wing, Steiner 1976 for the legs and Struhl 1977 for the probosis).

These results suggest that a cell's developmental potential may be progressively restricted by a sequence of determinative events starting at cellular blastoderm and continuing throughout larval development (Garcia-Bellido 1975).

This view of the functional significance of compartmentalization is supported by the phenotypes of some of the class of mutation known as *homeotics* (Bateson 1894, Postlethwait and Schneiderman 1973). In homeotic mutants a characteristic portion of the adult structures are replaced by a set of structures normally found elsewhere in the fly, eg., in *bithorax* the anterior of the haltere is transformed into anterior wing (Lewis 1955, 1963, 1964). It was observed that the transformations of some of the *bithorax* alleles (Garcia-Bellido 1975, Morata and Garcia-Bellido 1976) and of the mutant *engrailed* (Garcia-Bellido and Santamaria 1972) respected compartment boundaries previously defined by clonal analysis. These observations suggest that the cells in different compartments have activated different sets of genes. Such differential gene activity is expected if the cells in different compartments have selected different developmental pathways. This supports the hypothesis that the events reflected by the formation of compartmental boundaries are determinative events.

A quite different hypothesis as to how *Drosophila* imaginal disc cells decide which adult structure they will form has been proposed based on the results of disc regeneration experiments. When fragments of mature imaginal discs are implanted into late third instar larvae, the fragments undergo metamorphosis along with their host, and form vesicles of adult cuticle in the adult abdominal cavity (Ephrussi and Beadle 1936). If the cells in the fragment do not undergo cell division before metamorphosis, the adult structures produced by the fragment are always a specific part of the adult structure. Several investigators have prepared 'fate-maps' showing the locations of the precursor cells of specific adult structures by observing the structures formed by defined fragments of imaginal discs (see review by Gehring and Nöthinger 1973, also, Ouweneel et al. 1973, Bryant 1975). For example, Schubiger (1968) was able to map the locations of the precursor cells of a set of markers in the prothoracic leg disc. Even markers consisting of a single bristle (EB, BH^-) could be mapped.

These results indicate that late in the third larval instar the imaginal discs show a mosaic determination. That is, individual cells are committed to form specific adult structures. This is consistant with the hypothesis of a sequence of determinative events throughout development. However, when fragments were cultured under conditions where cell divisions occurred a different result was obtained. They sometimes formed structures normally produced by cells in a different portion of the same disc (regeneration), they sometimes produced two sets of the structures differentiated without an intervening period of cell proliferation (duplication) or, rarely, produced structures normally formed by a different imaginal disc (Hadorn 1965, 1968, Schubiger 1971, Bryant 1971, Strub 1977 a,b).

Hadorn (1965, 1968) studied the behavior of disc fragments cultured for long periods by repeated transfers to fresh adult hosts. He was able to demonstrate that such fragments usually formed structures appropriate to the imaginal disc involved. However, occasional changes to other discs occurred, which he termed "transdetermination".

Schubiger (1971) observed that fragments of the first leg disc did not duplicate and regenerate at random when cultured for 5-10 days prior to metamorphosis. Those fragments which contained the upper medial quarter of the disc regenerated while all others duplicated. This has recently been reconfirmed by Strub (1977 a,b) who also observed that the upper medial quarter of the first leg disc could regenerate all of the leg structures.

All of the cells in the upper medial quarter of the prothoracic leg disc are in the anterior compartment. However, when cultured, the descendents of these cells produce posterior structures. This indicates that cells are not limited to regenerating those structures in one compartment. These results indicate that, according to the operational definition of determination the committment to a particular compartment is not a determinative committment. It is worth noting that the anterior/posterior compartment boundary is established prior to the determination to form a specific imaginal disc. Nonetheless, cells only rarely produce structures formed by a different disc when cultured. This implies that there may be inherent differences in the stability of the different committments made by cells throughout development, and, that the stability of any one committment is not necessarily correlated with the stage at which it is made.

The observations made on regeneration of disc fragments led Bryant (1971) to propose that the ability of an imaginal disc cell to regenerate

is controlled by its position in a gradient of "developmental capacity". He proposed that each cell in the disc can regenerate those structures produced by cells with a lower gradient value, but not those with a higher value. Whenever a disc fragment is cultured *in vivo*, the cells at the cut edge are proposed to regenerate all structures produced by cells lower in the gradient. The cells at the cut edge of the fragment containing the high point of the gradient will thus replace the missing structures leading to regeneration. In the reciprocal fragment the same growth will produce a second copy of those structures already present, giving a duplication. According to this model there is no difference between the growth leading to a duplication and a regeneration. Since the upper medial quarter of the leg disc can regenerate all of the lateral structures, this portion was postulated to contain the high point of the gradient.

Using a different technique for generating the imaginal disc fragments, Postlethwait and Schneiderman (1973) investigated the regenerative ability of mesothoracic leg disc cells. They induced cell death in the discs by exposing young larvae to 1000 r of X-irradiation. They observed that in the trochanters and femurs of 30 cases of mesothoracic leg duplication the medial markers were often deficient and the lateral markers often duplicated. This result can also be explained by a single gradient in the leg, high medial and low lateral.

Bryant (1975) observed that when the wing disc is bissected and the half disc fragments cultured one half regenerates and the other half duplicates. According to the single gradient model the high point of the gradient is located in that portion of the disc which regenerates. Using a series of cuts he observed that the center of the disc was

common to all fragments that regenerated and was not present in any of the fragments that duplicated. This implied that several gradients or a single cone shaped gradient existed in the wing disc, high in the center and low at each edge. However, when this center portion was cut free from the edges and cultured it failed to regenerate and instead duplicated. This cannot be explained by the single gradient model.

Likewise some results obtained from grafting experiments in amphibian and cockroach legs cannot be explained by the single gradient model. When a distal leg graft is attached to a proximal stump so that no discontinuity exists then no regeneration occurs. However, when such a graft is made in which either the dorso-ventral or medio-lateral axes of the graft are reversed with respect to the stump (as occurs in grafts of right legs on left stumps) then a series of outgrowths occur from the stump/graft intersection. These 'supernumerary' growths contain a set of all of the leg structures distal to the level of the graft and are disposed in mirror image symmetry to the original leg (see Huxley and DeBeer 1934, Rose 1964, 1970, Hay 1966 for review of the amphibian limb, see Bohn 1965, 1972, Bullière 1970, and French 1976 a,b for review of the cockroach leg). In a series of recent studies using grafts between cockroach strains with different colored cuticle, French (1976 a,b) has reconfirmed that these supernumerary limbs show a mirror image symmetry (see Bateson 1894) and he has demonstrated that the cells of both the graft and the host participate in the formation of the supernumerary limbs. He has also confirmed the observation made earlier that when the graft and stump are from different portions of the leg, intercalative growth sometimes occurs which restores the complete leg, or generates a mirror-image duplication for a

portion of the leg. This intercalation always has the effect of removing a proximal-distal discontinuity, even at the expense of conserving constant limb polarity.

A similar type of intercalation occurs in *Drosophila* imaginal discs. When two fragments from opposite sides of a wing disc are mixed together, cultured and then induced to undergo metamorphosis, structures normally produced by cells in the center of the disc are found (Haynie and Bryant 1976). Either fragment when cultured alone duplicates, but the mixture of the two produces structures not normally formed by either fragment.

Several investigators have suggested that the results of these experiments can be explained using the concept of "positional information" developed by Wolpert (1969, 1971). Wolpert proposes that cells in a developing system are informed as to their location within the system by the localized values of global variables that uniquely specify the location of each cell. Cells are proposed to respond by differentiating in the proper patterns. The single gradient cannot be considered a model of positional information on logical grounds alone. To uniquely specify the position of every cell in the two dimensional sheet of cells that is an imaginal disc, at least two distinct gradients are required. One such model has been proposed by French et al. (1976) based on the observed patterns of symmetry in the supernumerary limbs in cockroaches and on the results of the amphibian and imaginal disc regeneration experiments. The authors postulate that two coordinate systems of positional information exist in the limb or imaginal disc, one radial and the other circular (Figure 1). They propose that cells confronted by a discontinuity in circular values always respond by intercalative

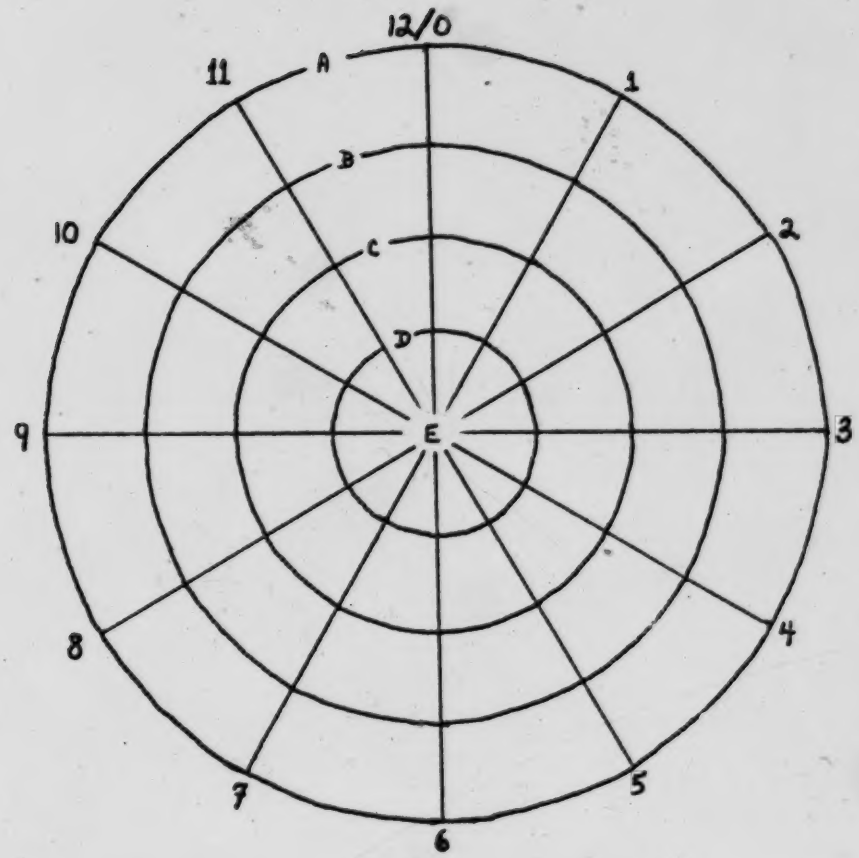


Figure 1 A diagram of the French, Bryant, and Bryant polar coordinate system showing the radial (A-E) and circular (0-12) positional information sequences. Redrawn from French, Bryant, and Bryant (1976).

growth that fills the gap via the shortest route. In an example given in their paper, an inserted graft that causes a confrontation between cells with circular values 4 and 8 in the cockroach leg induces intercalative growth of the structures with values 5, 6 and 7, thus removing the discontinuity via the shortest route.

They also propose that whenever a complete set of circular values is generated at any radial level, all structures distal to this level are regenerated. This accounts for the regenerative behavior in amphibian and cockroach legs. At the level of the cut a complete circle of values is present in the cells at the proximal and distal cut surfaces. The confrontation of these values during wound healing results in the initiation of distal regeneration. In the case of the supernumerary growths observed in amphibians and cockroaches, the confrontation of opposite circular values, due to the missaligned axes, at the stump/graft intersection is proposed to first stimulate intercalary growth which produces complete sets of circular values around the points of maximum difference between the stump and the graft. Then, these complete circles initiate the regeneration of a complete set of distal structures, producing the supernumerary leg. It is important to note that the patterns of symmetry in these supernumerary limbs will depend on the location of the confrontations between the graft and the stump that produce the complete circle. As shown by French et al. (1976) the model can successfully explain the location and symmetrics of the supernumerary limbs resulting from all combinations of graft/host confrontations which have been tested.

The polar coordinate model can account for the observed behavior of imaginal disc fragments cultured *in vivo*. In the wing disc, the

duplication of the central fragment is proposed to be the result of the distal transformation rule. The center of the wing disc is proposed to be equivalent to the distal tip of the leg. Thus when the edges of the central fragment come together during the wound healing process, a complete set of circular values is generated which initiates a regeneration producing a second copy of the structures present in the fragment. The regeneration behavior of wing disc fragments when mixed together is proposed to be the result of intercalation. Cells in the fragments are confronted with a discontinuity when mixed and they respond by regenerating those structures with intermediate values. To account for the observation that the upper medial quarter of the first leg disc can regenerate all of the leg structures, the authors propose that the circular values are not evenly distributed in this leg disc, and that half of these values are contained in the upper medial quarter of the disc. Thus intercalation by the shortest route in a fragment which contains the upper medial quarter of the disc will always result in regeneration, and in any other fragment will produce a duplication (see French et al. 1976, Figure 8).

The polar coordinate model thus fulfills the two requirements of a successful model. First, it explains all of the observed data with a minimum of *a priori* assumptions. Secondly, it makes predictions which can be experimentally tested.

Two views of the process whereby *Drosophila* cells become specified as to which adult structure they will form have been presented above. The clonal restriction data suggests that cells become progressively committed to form specific adult structures by a series of decisions. Each cell remembers the result of the decisions made by its ancestors

and passes on to its progeny the result of any decisions it makes. The positional information model based on the results of regeneration experiments postulates that which adult structures are formed by an imaginal disc cell depends on the cell's location in the mature disc. The location of each cell is uniquely specified by global systems of positional information in the disc. Each cell interprets this information and responds by differentiating a specific adult structure during metamorphosis.

One possibility for the reconciliation of these two views is that positional information controls the spatial organization of determinative events in the embryo and imaginal discs, and, that these decisions are necessary steps in the interpretation of positional information. Thus at several stages of development cells might make decisions based on their position which serve to restrict the developmental potential of their progeny.

The observations that cells can regenerate structures they would not normally form can be reconciled to this view by postulating that at the initiation of regeneration cells become de-determined, i.e., they "forget" the decisions made by their ancestors, and that during the process of regeneration the cells repeat the sequence of decisions which occur in normal development but in response to a new pattern of positional signals. The rules which govern the establishment of this new pattern are presumed to be inherent in the organization of the original system of positional information. Thus regeneration or duplication would be a reiteration of normal development. This hypothesis might be tested by analyzing the intermediate stages of growth of pattern duplications and comparing them with those of normal development. Studies

of the development of normal imaginal discs have provided information about two aspects of development which are germane to the hypothesis: (i) the number of cells present in the disc at different stages and (ii) the formation of compartment boundaries.

These aspects of development have been studied using the technique of clonal analysis. Up to now the application of clonal analysis to studies of the growth of duplications of imaginal discs was technically very difficult, due to the large amount of effort required to generate a significant number of cases. In a demonstration of the utility of genetics in the study of development, Russell (1974) has recently developed a system in which such studies can be made. He isolated a series of temperature sensitive, cell autonomous lethal mutations in *Drosophila*. One of these, *l(1)ts726* (referred to in this thesis as *ts726*) was a 29° restrictive, 22° permissive allele at the *suppressor of forked* (su(f) 1-65.9) locus. When *ts726* larvae are subjected to a sublethal pulse of the restrictive temperature, cell death is induced in the imaginal discs. The resulting adults have a high frequency of deficiencies and duplications for structures in the head and the mesothoracic leg (ibid). Histological observations indicate that the cell death is clustered in these discs (Clark 1976, Clark and Russell 1977) suggesting that these patches of cell death are creating *in situ* disc fragments analogous to those produced by surgery.

In the present thesis the *ts726* system will be used to induce duplications of the mesothoracic leg. The work is designed to investigate two main points. First, the predictions of the single gradient and polar coordinate models will be tested by a careful phenotypic analysis of a large number of mesothoracic leg duplications. Secondly, an attempt will

be made to determine whether the initiation and intermediate stages of growth of duplications are similar to those of normally developing legs. This will be done by comparing the results of somatic clonal analyses of duplications with the results of similar analyses done on normal legs. Similar results would provide strong evidence for the hypothesis that pattern duplications are formed by a reiteration of normal development.

MATERIALS AND METHODS

Drosophila Stocks and Mutations

The mutations, special chromosomes and stocks of *Drosophila melanogaster* used are listed in Table 1. Except when otherwise stated a detailed description of the mutations and special chromosomes can be found in Lindsley and Grell (1968). All strains used were derived from stocks kept in this laboratory with the exception of *Dp(3:1)mwh*$^{+}$ a duplication carrying the wild type allele of multiple wing hairs [(*mwh* [3-0.0]) distal to *y* on the X chromosome. This duplication was kindly supplied by Dr. J. Merriam.

Culture Conditions

Composition of the Medium

Drosophila were reared in 1/2 pint glass bottles containing approximately 50 ml of culture medium or in shell vials containing approximately 8 ml of medium. The yeast-agar medium of Nash and Bell (1968) was used throughout. This consisted of 10 g yeast, 10 g sucrose, 1 g agar, 1 ml propionic acid and 10 µg chloramphenicol per 100 ml of distilled water. The chloramphenicol and propionic acid are both added to the freshly cooked medium when it has cooled to 70° (all temperatures are given in degrees Celsius), but prior to pouring into individual culture containers. In some of the experiments the amount of chloramplenicol was varied from 0 to 100 µg per 100 ml of distilled water. In these cases the medium was prepared in quantity and poured into 500 ml flasks for the addition of the chloramphenicol. Care was taken to ensure proper mixing prior to pouring the medium into individual containers.

Table 1 Mutations, Special Chromosomes, and Stocks of *D. melanogaster*

Mutations	Map Position	Phenotype	Reference
$su(f)^{ts726}_{y}$	1-65.9 1- 0.0	ts lethal (see text) adult body and bristles yellow	Russell (1974) Lindsley and Grell (1968)
f	1-56.7	bristles forked and gnarled	"
f^{36a}	1-56.7	the most extreme f allele	"
v	1-33.0	vermilion eye color	"
mwh	3- 0.0	cell hairs of wing and other surface areas multiple, aristae also affected	"
sn^3	1-21.0	bristles singed, female fertile allele	"
w	1- 1.5	white eye color	"
e	3-70.7	adult body black, heterozygote intermediate	"
red	3-53.6	red malpighian tubules eyes brown	"
$M(3)i^{55}$	3-28.9	a strong *Minute*	Garcia-Bellido *et al* (1973)

Chromosomes	Description	Reference
y^+Y	a duplication carrying y^+ and ac^+ on the tip of Y^L	Lindsley and Grell (1968)
B^3 Y	a duplication carrying $su(f)^+$ on the tip of Y^L	"
C(1)DX	a reversed acrocentric compound X chromosome which is heterozygous for In(1)d149	"
Dp(1:3)sc^{J4}	the aneuploid segregant from T(1:3)sc^{J4}/+	"
TM6	Third Multiple 6, a multiply inverted third chromosome	"
FM7a	First Multiple 7a, a multiply inverted X chromosome	J. Merriam (1969)
Dp(3:1)mwh^+	a duplication of distal 3R on the tip of the X chromosome isolated by recombination of a 3:Y^S translocation with an $\overline{XY}$ chromosome	J. Merriam (personal communication)

Table 1 continued

Stocks

C(1)DX/B^SY/*y v f 726*

C(1)DX/y^+Y/*w* sn^3 *l(1)ts538*

Dp(3:1) *mwh*$^+$ *y v f 726*/FM7a; *mwh/mwh*

w sn^3 *726*/B^SY; *mwh e/mwh e*

y w f 726/Y; *mwh/mwh*

y v f 726/Y; Dp(1:3) sc^{J4} y^+ *M(3)i*^{55}/TM6

Mating and Egg Collection

Accurately staged, synchronous cultures of *Drosophila* were obtained for experimental manipulation from timed egg layings. Parental females were collected as virgins, mated and the adults held for 3 days prior to beginning the egg collections. During this holding period the adults were transferred to fresh medium daily to prevent egg retention by the females. Egg collections were made by exposing the parental adults to fresh medium for 4, 6, 12 or 24 hours as noted in each experiment. Larval ages and treatment times are given in hours after the midpoint of the egg collection period.

Experimental Techniques

Temperature Treatments

Sublethal 29° treatments were used to induce cuticular abnormalities in the *ts726* experimental subjects. The temperature regimen for all such experiments was as follows. Eggs were collected at room temperature, less than 25°. Immediately following the collection the eggs were transferred to a controlled temperature incubator maintained at 22°. Each egg collection batch was incubated at 22° for a predetermined period. For the 29° treatment each batch was transferred to an adjacent 29° incubator for a predetermined number of hours. The collection batches were then returned to 22° to complete development. The times of incubation are given in each individual experiment, and were controlled to within ±15 minutes and the temperatures to within ±1° of the stated values.

For convenience the temperature conditions under which the larvae were reared are indicated by an abbreviated notation. For example, an egg collection batch incubated at 22° for 72 hours from the midpoint of

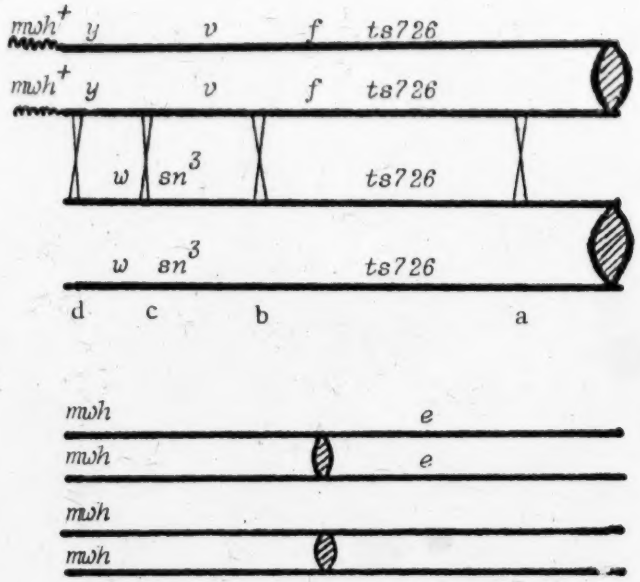
the egg collection and then transferred to 29° for 48 hours would be described as having received a 72 hour 22°/48 hour 29° treatment.

Radiation Treatments

Radiation treatments were given to developing larvae either to induce somatic recombination (Becker 1957) or in studies of the effects of radiation on development. Gamma radiation was used, and was given by exposure to a Cobalt^{60} source which at the time had a dose rate of 2500 rads per minute (Dr. S. Quah pers. comm.). Individual doses were controlled by varying the length of exposure, using an automatic timer. The larvae were irradiated in the original egg collection containers as the large numbers of larvae being treated made the transfer to new containers impractical. No correction for the type of container was made, as tests of the 1/2 pint bottles indicate that they absorb less than 1% of the radiation (Dr. A. Noujaim pers. comm.). It was assumed that the shell vials, being of lighter construction than the bottles, would absorb even less of the radiation.

Somatic Recombination

Two genetic systems were used in the somatic clonal analyses reported in this thesis. First, recombination was induced between the X chromosomes in *mwh*$^{+}$ *y v f36a ts726/w sn3 ts726;mwh/mwh e* females. Such a recombination in an imaginal disc cell will often result in the two daughter cells having a different genotype (Stern 1936). The exact differences depend on the location of the recombination event along the X chromosome, as shown in Figure 2. The descendants of these daughter cells will form two clones of marked tissue in the adult. According to the mitotic recombination map published by Becker (1974) the majority (74%) of these clones will be twin spots (Stern 1936, 1968), in which the descendants of



	Clones resulting from each recombination type		percent
a.	*y f- mwh sn*^{3}	twin spot	74
b.	*y - mwh sn*^{3}	twin spot	17
c.	*y - mwh*	twin spot	9
d.	*mwh*	single	>1

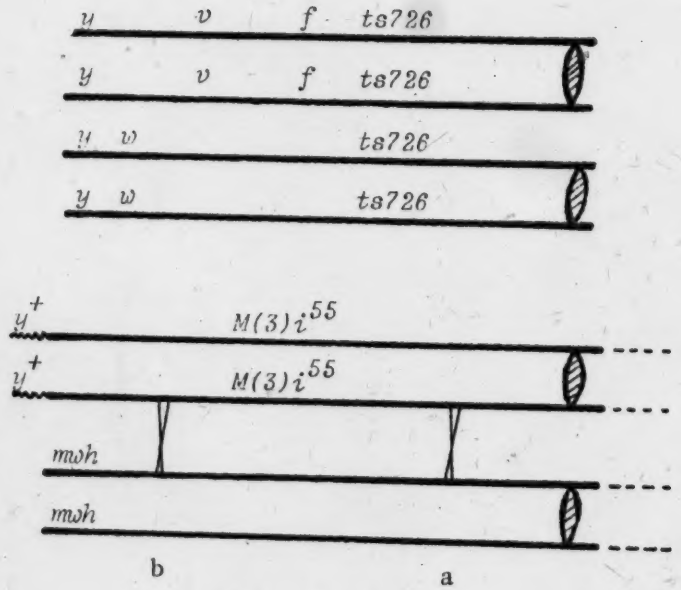
Figure 2 A diagram showing the somatic cell clones produced by radiation-induced recombination between the X chromosomes in a *726* female. The location of the recombination along the chromosome determines whether (a) *mwh sn*^{3} *y f* twin spots (b) *mwh sn*^{3} *y* twin spots (c) *mwh y* twin spots, or (d) *mwh* single spots are produced. The percentage of the total clones expected in each class is given, based on the induced mitotic recombination map of Becker (1974).

each daughter cell form a separate, visible spot. One spot will contain both trichomes marked with multiple wing hairs and bristles marked with singed (*mwh sn*), and the other bristles marked with yellow and forked (*y f*). A description of the other 26% of the clones is given in Figure 2. It is expected that not all of the single spots will be scorable. This is because the mesothoracic leg is not uniformly covered with trichomes and bristles, i.e., a *y f* spot in an area of the leg containing no bristles cannot be scored.

The second genetic system used was a heterozygous *Minute* system (Garcia-Bellido et al. 1973, Morata and Ripoll 1975). Recombination was induced in the right arm of the third chromosome of *y w ts726/ y v f ts726;Dp(1:3)sc^{J4}, y^{+} M(3)i^{55}/mwh* females and *y w f ts726/Y; Dp(1:3)sc^{J4} M(3)i^{55}/mwh* males. As shown in Figure 3, such a recombination in an imaginal disc cell may lead to the production of a single spot clone. One of the two daughter cells will be homozygous *Minute* and will die. The descendants of the surviving cell will form a single spot containing both trichomes marked with multiple wing hairs and bristles marked with yellow (*mwh y*). The cells of this clone, being wild type for *Minute*, will grow at a faster rate than the heterozygous *Minute* background cells. Thus the clone produced in the adult will be large (ibid).

Scoring of Mesothoracic Leg Abnormalities

The mesothoracic legs from treated *ts726* adults were scored for the presence of cuticular abnormalities and/or somatic cell clones. Eclosing adults were collected for scoring from each egg collection batch at daily intervals. The eclosed adults were stored in 70% ethanol. When no more of the adults were likely to eclose, the uneclosed



Clones resulting from each recombination type		percent
a. *y mwh* M^+	single	81
b. *y mwh*	single	19

Figure 3 A diagram showing the somatic clones produced by radiation-induced recombination in the right arm of the third chromosome in a *726 Minute* female. The location of the recombination event determines whether (a) *y mwh Minute+* (b) *y mwh* or (c) *y* single spots are produced. The percentage of the total clones expected in each class is given, based on the induced mitotic recombination map of Becker (1974).

pharate adults were washed from the sides of the container in a 7% sodium chloride solution. They were then collected by straining, washed in distilled water and stored in 70% ethanol.

A sizable proportion of the *ts726* pharate adults fail to eclose following a 29° treatment. The data from these adults was included in the results in one of two ways. Either all of the pharate adults were dissected from the pupa cases and scored along with the eclosed adults, or, when the large numbers made this impractical, a sample of the pharate adults were selected from each treatment batch, dissected and scored.

The adults were first separated according to genotype and were counted under a Wild M-5 stereo dissecting microscope at a magnification of 25-50x. Then, the *ts726* adults were cooked in 1N NaOH to remove the inner body parts. These cooked adults were then scored under the dissecting microscope. Those adults with abnormalities of the mesothoracic leg were dissected with fine iridectomy scissors and/or electrolytically sharpened tungsten needles. The two mesothoracic legs from each fly were mounted together between glass coverslips in Gurrs water mounting medium for scoring under a Wild M-20 compound microscope at a magnification of 200-400x.

The number of *ts726* adults surviving to at least the pharate adult stage was compared with the number of control adults to give an indication of the effect of the treatments on survival. The ratio of *ts726* adults/control adults is referred to as the relative survival of *ts726* adults, eg., 100% relative survival means as many *ts726* as control adults reached at least the pharate adult stage. In those batches in which all of the uneclosed pharate adults were not scored the total number of *ts726* adults was estimated using the fraction of the *ts726*

adults in the sample of pharate adults. The calculation was done as follows. Let A represent the number of *ts726* pharate adults in the sample, B the total number of pharate adults in the sample, C the total number of pharate adults in the batch, D the number of eclosed *ts726* in that batch and E the estimated total number of *ts726* adults in the batch. Then:

$$\left(\frac{A}{B}\right) C + D = E$$

The frequency of duplications of the mesothoracic leg in 29° treated batches was calculated as a percentage of the total legs. In those cases where all of the pharate adults were not scored, the frequency of leg duplications in the sample of pharate adults scored was used to estimate the total frequency of duplications. The calculation was done as follows. Let F represent the number of leg duplications in the sample of pharate adults, G the number of duplications in the eclosed adults and H the percentage of duplications in all of the mesothoracic legs of that batch. Then:

$$\frac{\left\{\left(\frac{F}{2A}\right) 2C + G\right\}}{2E} \times 100 = H$$

It is important to note that the relative survival frequency is a frequency of *ts726* adults while the duplication frequency is a frequency of legs.

Imaginal Disc Transplantation

In addition to the analysis of mesothoracic legs which had completed development *in situ*, a series of imaginal-disc fragments were transplanted and their regeneration/duplication pattern studied after *in vivo* culturing.

Disc Preparation

Imaginal discs were isolated for transplantation from mature, third instar larvae that had left the culture medium and were crawling on the sides of the container. These larvae were sexed by gonad size under the dissecting microscope (Demerec 1950) and the male *ts 726* larvae were then dissected in a modified insect ringers (Chan and Gehring 1968), supplemented with 100 iu/ml of penicillin, 100 µg/ml of streptomycin and 100 µg/ml of kanamycin (Lee and Gaerhardt 1973). The mesothoracic leg disc were identified by their position in the larva (Demerec 1950) and were dissected from the cephalic complex using fine pointed forceps and electrolytically sharpened tungsten needles. The discs were then transferred to a glass depression slide in a drop of fresh ringers. They were then carefully examined for damage sustained during dissection.

Undamaged discs were cut into fragments in one of two ways. Some discs were cut into halves by pressing them against the glass slide with a tungsten needle. Others were cut using a blunt, hollow glass needle. In this case the needle was used as a punch to remove a small semi-circular fragment from the edge of the disc. Using needles with different diameters allowed the removal of different sized fragments. The half disc fragments and the larger fragments from the second type of cut were then drawn into a sharpened glass needle for transplantation.

These needles were prepared by drawing a fine glass capillary pipet (Drummond "Microcaps" 100 µl) over a flame and then sharpening the point with carborundum powder or by fracturing (Ursprung 1967). A series of needles were made with tip diameters ranging from approximately 50-150 µm. Once sharpened, these needles were rinsed in 70% ethanol and were then coated with .1% silicone solution (siliclad) to keep the disc fragments

from sticking. Disc fragments were drawn into the needle by suction, using a mouth pipette. Care was taken to use a needle with a diameter approximately 1/2 that of the fragment. This was small enough to help to prevent the loss of the fragment by drawing it too far into the needle and large enough to reduce fragmentation during implantation.

Hosts

Two types of hosts were used in these experiments. The disc fragments were either implanted into mature third instar *mwh red e* larvae for metamorphosis or were implanted first into the abdomen of an adult *mwh red e* female for a period of growth. Cell proliferation occurs in the disc fragments under these conditions but differentiation does not occur (Bodenstein 1957, Schneiderman and Gilbert 1959 for review). The disc fragments were recovered after the *in vivo* growth period and were implanted into mature larvae for metamorphosis.

The mature larvae used as hosts were collected, washed in distilled water, rinsed briefly in 70% ethanol and then rinsed in insect ringers. Next the larvae were etherized for 1-2 minutes and then attached to a glass slide with double sided tape for implantation. The disc fragments were implanted by injection using the hollow glass needles described previously. After implantation the larval hosts were washed free from the tape with insect ringers and placed in shell vials containing fresh medium to complete development.

The disc fragment undergoes metamorphosis with the host larva and forms a vesicle of adult cuticle in the adult abdomen (Ephrussi and Beadle 1936). These vesicles were dissected from the hosts 1 day after eclosion, or from the pharate adult hosts that failed to eclose. The vesicles were rinsed briefly in cold 5N NaOH to remove any host tissue,

were washed in 70% ethanol and were then cut open with tungsten needles to aid in scoring. After this preparation the vesicles were mounted between glass coverslips in Gurr's water mounting medium and scored under the compound microscope.

Morphological Markers of the Mesothoracic Leg

The morphological markers scored in the mesothoracic legs and in the vesicles are shown in Table 2. These markers are the same as those reported by Hannah-Alava (1958) or by Schubiger (1968) with the following exceptions. The 1GSt and 2GSt of Schubiger were scored as individual rows; 1St7, 2St7, 1St6 and 2St6. The TST, TSc3, TSc2, mSc1, mSc2 and 3Sc2 are newly described (but see Postlethwait and Schneiderman 1973).

Table 2 Morphological Markers Scored in the Mesothoracic Leg[1]

	Thorax
ST	Sternal macrochaete
ThB	Thoracic microchaetae
SP	Sternopleural macrochaete
	Coxa
St8	8 sensilla trichodea
BH	Hairy island bristle
St4	4 sensilla trichodea
	Trochanter
1St7	row of 3 sensilla trichodea
2St7	row of 4 sensilla trichodea
1St6	row of 3 sensilla trichodea
2St6	row of 3 sensilla trichodea
St1	single sensillum trichodeum
Sc5	3 campaniform sensilla
EB	Edge bristle
Sc8	8 campaniform sensilla
Sc3	3 campaniform sensilla
St5	row of 5 sensilla trichodea
	Femur
Sc1	single campaniform sensillum
Sc11	11 campaniform sensilla
	Tibia
TST	4 large trichodea-like sensilla
TSc2	2 large campaniform-like sensilla
TSc3	3 large campaniform-like sensilla
PAB	Pre-apical bristle
AB	Apical bristle
Tsp	Tibial spurs flanking AB
	Tarsus
mSc1	Single medial metatarsal campaniform sensillum
mSc2	Pair of lateral metatarsal campaniform sensillum
3Sc2	Pair of lateral campaniform sensilla on 3rd segment
Un	Ungues
Em	Empodium
P	Pulvilli
AP	Unguitractor apodeme
Unp	Unguitractor plate

1. After Hannah-Alava (1958) Schubiger (1968); see text

RESULTS

The results are presented in three sections. The first deals with a description of the phenotype of the leg duplications induced using *ts726*. Samples of duplications were compared with unduplicated control legs and the patterns of abnormality defined. The second section deals with the effect of changing the 29° treatment conditions on the frequency of duplications in and on the relative survival of *ts726* adults. Three factors were tested; the length and timing of the 29° treatment, the effects of combining radiation treatments and 29° treatments and the effects of changes in the culture medium. The third section deals with the cellular parameters of the initiation and growth of leg duplications, investigated using the technique of somatic clonal analysis. I was particularly interested in establishing whether or not the patterns of cellular activity in the duplicate legs were similar to those of normal legs.

Morphological Investigations of the Duplication Phenotype

The first step in the investigation of the duplications induced by *ts726* was a morphological analysis of the duplication phenotype. It was important to determine how the duplicated legs differed from normal and whether there were any patterns within the abnormal phenotype that could be identified. Accordingly, a large sample of leg duplications which had been collected by Russell (Russell, Girton and Morgan 1977) were examined in detail.

Experimental Procedure

Russell mated large numbers of *C(1)DX, y f/y$^+$ Y* virgin females collected from a *C(1)DX, y f/w sn^3 l(1)ts538/y$^+$ Y* virginater stock to *y v f ts726/B^{S}Y* males to generate *y v f ts726/y$^+$ Y* experimental males

(called 726 males) and *C(1)DX/B^{s}Y* females (called control females) that served as an internal viability control (Russell et al. 1977). Eggs from this mating were collected over 4 hour intervals and were divided into batches for treatment. Two batches served as controls, one continuously incubated at 22° and the other at 29°. The remaining batches were each given a 48 hour 29° treatment at one of several times in development.

The resulting eclosed adults from each batch were collected and counted under the dissecting microscope and the 726 males with abnormalities were prepared for scoring under the compound microscope as described in the materials and methods. However, all of the uneclosed pharate adults were not scored in the same manner as the eclosed adults. Instead, the pharate adults were counted under the dissecting microscope and a sample of 50 726 males from each treatment batch was prepared for scoring under the compound microscope. This sample was selected without screening for abnormalities. The proportion of mesothoracic leg duplications in each sample was used to estimate the total number of duplications in each batch, as described in the materials and methods.

The frequencies of leg duplication in and relative survival of 726 males for a sample of the treatment batches are given in Table 3. The 29° control showed no survival of 726 males, indicating that *ts726* is a non-leaky lethal at 29°. The 22° control showed no duplications and slightly higher survival than the control females, indicating that *ts726* does not induce duplications or decrease survival at 22°.

There was a large variation in both the duplication and the survival frequencies depending on the time at which the 29° treatment was given. Duplications were induced by treatments beginning as early as 34 hours

Table 3 Survival and DFP Frequencies in Relation to Time of 29° Treatment[1]

Time Shifted to 29° (hr)	Number of Control Females	Number of 726 Males	Relative[2] Survival of 726 Males (%)	726 Pharate Males (%)	DFPs (#scored)	(%)[3]
30	1488	8	5	0	0	0
34	1691	134	8	7	2	1
38	1596	57	4	21	2	3
42	2170	349	16	11	28	4
50	623	488	78	26	155	25
58	1096	610	56	58	114	40
66	1582	887	56	59	196	47
74	868	472	54	35	243	38
82	1225	628	51	44	167	34
90	1603	1322	82	22	352	20
98	767	494	64	44	188	30
106	1222	466	38	62	42	10
114	1253	458	37	72	19	9
122	1236	344	28	95	5	3
130	1246	881	71	100	2	2
138	1572	1431	91	100	0	0
146	644	825	100	100	0	0
154	1091	887	81	96	0	0
162	1400	1241	89	87	0	0
170	581	696	100	34	0	0
Controls						
22°	1094	1384	100	-	0	0
29°	890	0	0	-	-	-

1. Data from Russell, Girton, and Morgan (1977) Table 2
2. Calculated as described in the Materials and Methods (p.26)
3. Calculated for each treatment by averaging the DFP frequencies among eclosed and pharate adult 726 males, weighted by the number in each category, as described in the Materials and Methods (p.27)

and as late as 130 hours. There was a broad peak of duplication frequency with the maximum (47%) occurring following the 29° treatment started at 66 hours. The relative survival of 726 males varied between 51-82% in the treatments showing a high (20%) frequency of duplications.

A Description of the Normal Mesothoracic Leg Phenotype

Before the abnormal leg phenotype could be determined it was necessary to define the normal leg phenotype. Accordingly, a sample of 170 of the 22° control legs were scored under the compound microscope. First, each leg was scored for the number of elements (sensilla, bristles, etc) in each of the 32 cuticular markers described in the materials and methods. The resulting string of numbers for each leg was entered onto a punched card for computer analysis. The mean number of elements in and the standard deviation of each marker were then determined and are shown in Table 4. As indicated by the small standard deviations, the number of elements in the markers was the same in most of the legs. Next, the position of each marker, the rows of bristles and the areas of the leg containing trichomes were indicated on a diagram of the adult leg (Figure 4). The overall design of this diagram follows that of Steiner, although the tarsus was not represented in his diagram of the mesothoracic leg (Steiner 1976).

Following the preparation of the diagram of the adult leg, a fate-map indicating the locations of the cells in the imaginal disc which will form each of the 32 morphological markers was prepared (Figure 5). This fate-map was produced by adapting the fate-map of the prothoracic leg disc published by Schubiger (1968) to the mesothoracic leg. In this adaptation the markers characteristic of the prothoracic leg were eliminated, those found in the same location in both

Table 4 The Number of Elements in the Morphological Markers in 22° Control Legs[1]

Marker[2]	Number of Elements	
	mean	standard deviation
ST	1.1	0.1
ThB	6.1	0.4
SP	2.0	0.2
St8	8.1	0.2
BH	1.0	0.1
St4	4.0	0.2
1St7	3.0	0.0
2St7	4.0	0.1
1St6	3.0	0.0
2St6	3.0	0.1
St1	1.0	0.1
Sc+5	5.0	0.1
EB	1.0	0.2
Sc 8	8.0	0.0
Sc3	3.0	0.1
St5	5.0	0.0
Sc1	1.0	0.0
Sc11	11.1	0.3
TST	3.9	0.4
TSc2	2.0	0.0
TSc3	3.0	0.0
PAB	1.0	0.2
AB	1.0	0.0
Tsp	5.0	0.7
mSc1	1.0	0.0
mSc2	2.0	0.0
3Sc2	2.0	0.0
Un	2.0	0.1
Em	1.0	0.0
P	2.0	0.0
Unp	1.0	0.0
Ap	1.0	0.0

1. Data from a sample of 170 22° control 726 males
2. See materials and methods for marker definitions

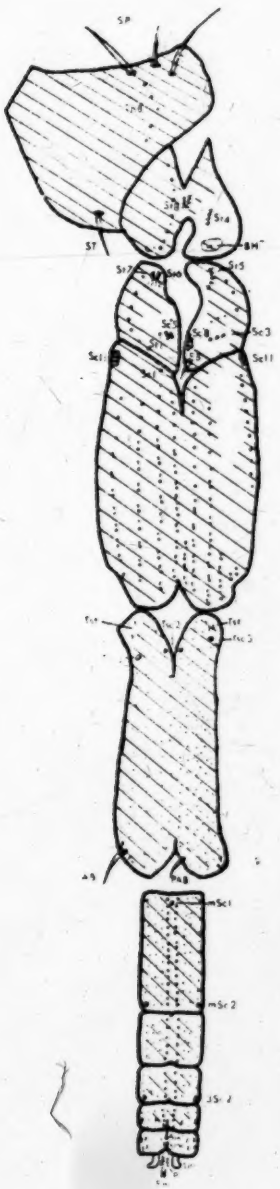


Figure 4 A standard map of the adult mesothoracic leg prepared by observing the legs from 170 22° control *726* females. The marker designations are given in Table 2.

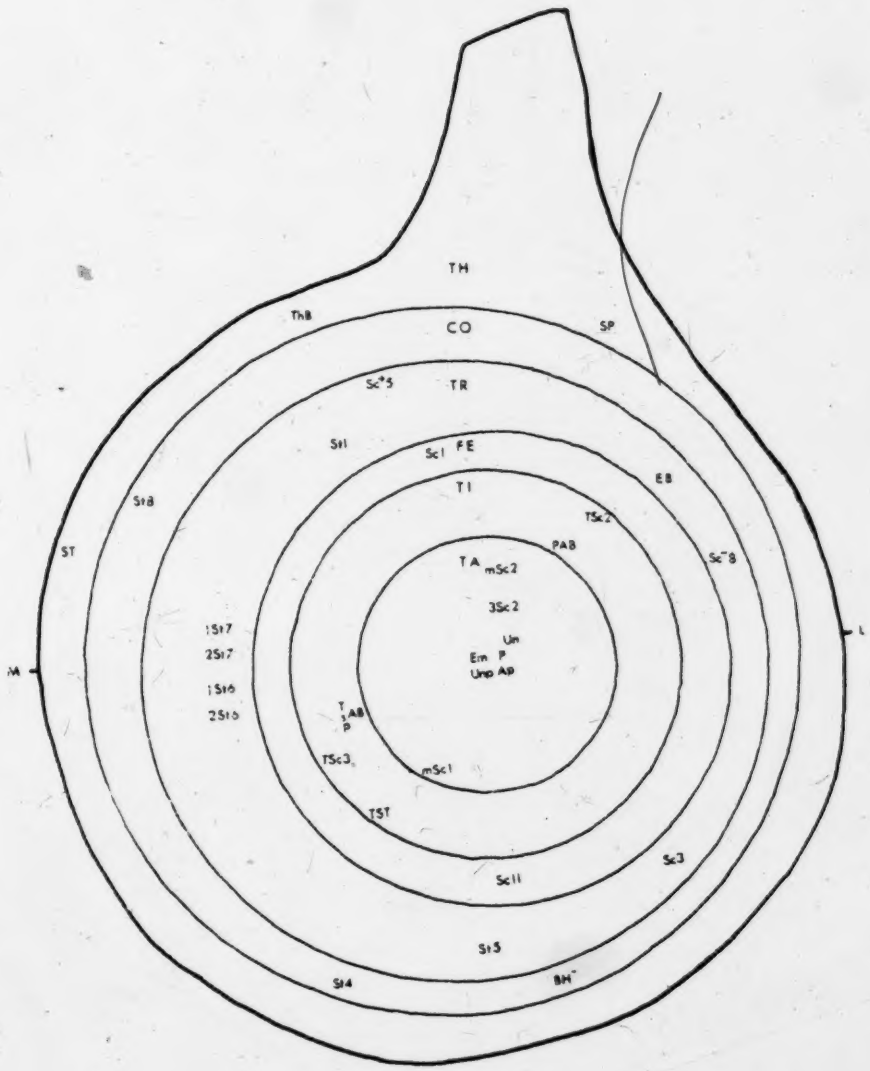


Figure 5 Fate map of the mesothoracic leg imaginal disc prepared by adaptation of the fate map of the prothoracic leg imaginal disc of Schubiger (1968) and revised using the results of an imaginal disc transplantation experiment (see text). The marker designations are given in Table 2.
m = medial L = lateral

adult legs were retained unchanged and those characteristic of the mesothoracic leg were positioned according to their location in the adult leg.

A Test of the Mesothoracic Leg Fate-Map

The resulting fate-map was tested and refined by an imaginal disc transplantation experiment. In this experiment a series of imaginal disc fragments generated by defined cuts were implanted into mature larval hosts. Such fragments metamorphose with the host and normally form only those structures which they would have formed *in situ* (Ephrussi and Beadle 1936, Schubiger 1968).

The same genetic stocks and mating scheme were used as in the previous experiment. Individual *72s* male larvae were selected for treatment from cultures continuously incubated at 22°. These males were dissected in insect ringers and their mesothoracic leg discs were removed, as described in the materials and methods. Each disc was cut along one of the 4 lines shown in Figure 6 a on a fate-map and 6b on a photograph of a mesothoracic leg disc. Each fragment was then implanted into a mature larval host from a stock bearing the markers *mwh*, *red* and *ebony*. Following metamorphosis of the host the vesicle of adult tissue formed by the disc fragment was recovered and prepared for scoring as described in the materials and methods.

The frequencies with which scorable vesicles were recovered for each of the disc fragments are given in Table 5. On the average 22.8% of the implantations into mature larval hosts resulted in a scorable vesicle of adult cuticle.

An example of the structures formed in a 2B disc fragment vesicle is shown in Figure 7a. This figure shows the trochanter and upper

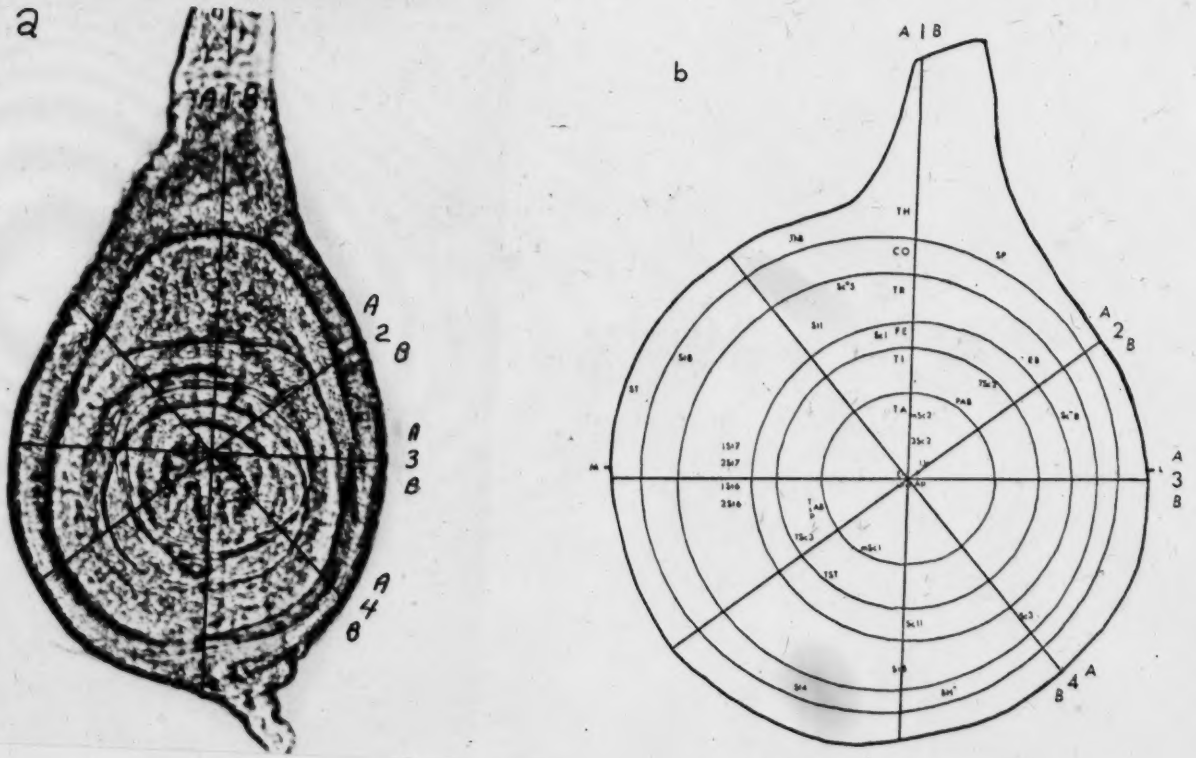


Figure 6 Locations of the 4 cuts made in the imaginal disc fate map test experiment. Each half of the disc was implanted into a mature larval host. The cuts are shown on (a) a photograph of a mesothoracic leg disc and (b) on a fate map.
M = medial L = lateral

Table 5 The Frequency with which Implants were Recovered after Injection into Mature Larval Hosts

Fragment	Attempts	Implants	Success (%)
1A	25	5	
B	25	6	
2A	27	6	
B	27	6	
3A	23	7	
B	23	4	
4A	26	4	
B	26	6	
Total	202	46	22.8

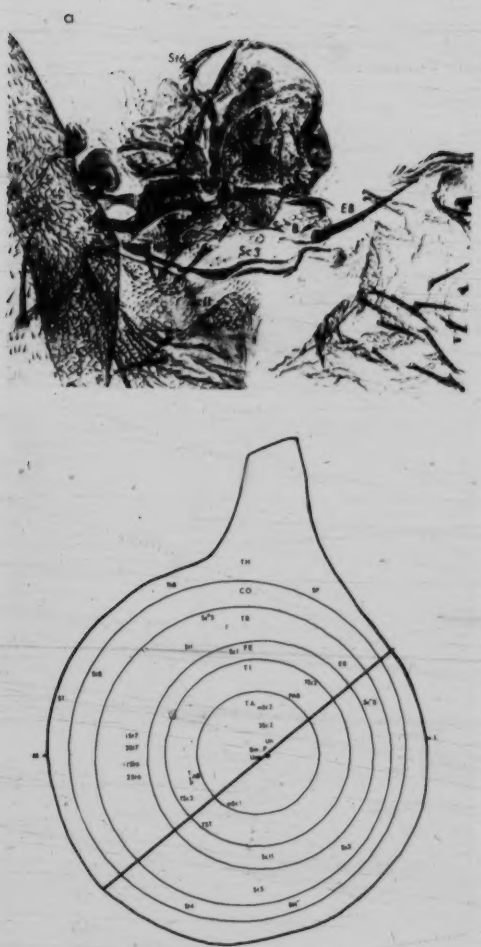


Figure 7 An example of a vesicle resulting from the implantation of a 2B disc fragment into a mature larval host.
(a) markers present in the trochanter
(b) a fate map showing the location of the cut

femur of a 2B fragment. The structures formed are indicated in Figure 7b on a fate-map. Only those structures from the lower half of the disc are present, as expected.

The results from the total series of 1/2 disc implants are summarized in Figure 8. The frequency with which each marker was found in each sample of vesicles is indicated. In this figure the markers were scored as being present if any portion could be scored. The markers present in each individual vesicle are given in Appendix A. In general, each fragment forms only those structures in half of the disc, as expected. There are, however, instances of overlap in which markers close to the line of the cut are found in both fragments. These may be due to inaccuracies in the position of individual cuts and perhaps also to the occasional bisection of the *anlagen* of markers with multiple units. The results agree well with the results of Schubiger (1968) for those markers included in his map of the first leg disc.

The location of each of the characteristically mesothoracic markers was determined by observing which of the two fragments generated by each cut contained that marker. For example, the ST is found in fragments 1A, 2A, 3A and 4B. This indicates that the *anlagen* of the ST is in the lower half of the upper medial quarter of the disc, i.e., that area common to all four of these fragments. Each mesothoracic marker was located in this fashion, and the results used to revise the fate-map (the revised map has been shown throughout). No material differences were observed between the location of a marker by its position in the adult leg, and its location determined above.

A Description of the Duplication Phenotype

An example of a leg duplication induced by *ts726* is shown in Figure

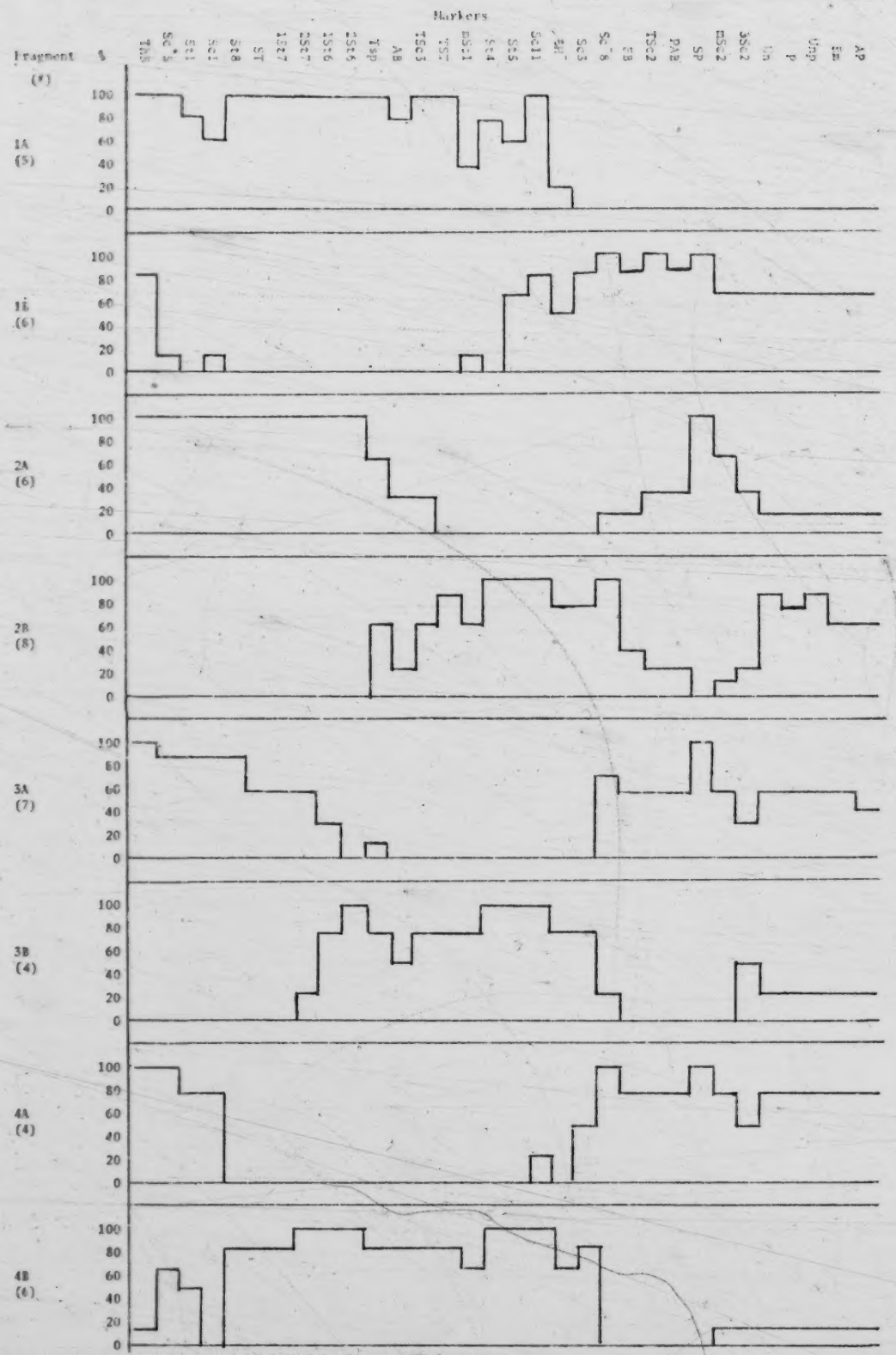
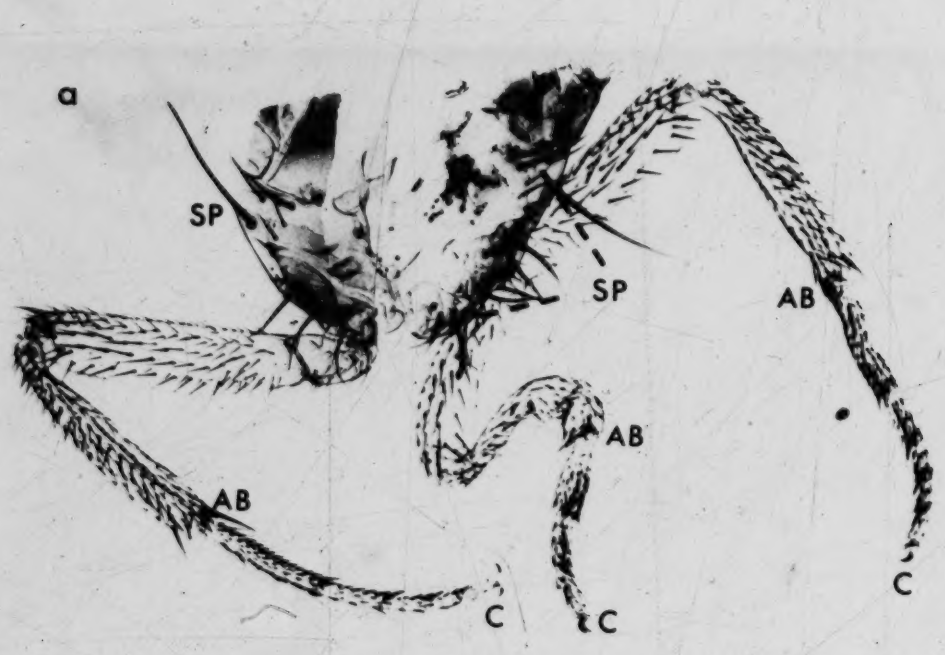


Figure 8 A diagram indicating the percentage of cases in which a marker was found in half disc fragments after metamorphosis. In each case the marker was scored as being present if any portion of it was found. The number of cases is indicated for each fragment type; see Figure 5 for an explanation of the fragment designations.

9a. The two legs shown are from a single *ts726* fly. One leg, the right leg in this case, is morphologically normal. The other leg is partially deficient and partially duplicated, i.e., some of the markers of the mesothoracic leg are not present, and the remaining markers are present twice. The two areas deficient and duplicated are indicated in Figure 9B, on a fate-map. Legs containing such deficiency-duplications will be referred to in this thesis as *DFP's*. As can be seen from the examples shown in Figure 10, the duplicated sets of markers in a DFP show a mirror-image symmetry.

The two portions of each DFP were separately classified on the basis of this difference in symmetry. Those portions showing ipsilateral symmetry, eg. "left-handed" symmetry in a DFP on the left side of the fly, were classified as *Original* portions. Those showing contralateral, eg. "right-handed" symmetry in a DFP on the left side of the fly were classified as *Duplicate* portions. This classification was partly based on data to be presented later and partly on the results of earlier studies on duplication in imaginal discs (Wildermuth 1968 a,b, Postlethwait et al. 1971, Ulrich 1971, Nöthinger 1976). The results of these studies indicate that the duplicate is formed by a small number of cells. This suggests that duplication in imaginal discs occurs by epimorphosis (Morgan 1901). That is, that the new growth stimulated by the wounding of the disc produces the second set, or duplicate structures. If so, the first set would be formed by the cells already present prior to the wounding. If the organization of these "original" cells were to remain unchanged, they would be expected to form those structures with ipsilateral symmetry.

In addition to the difference in symmetry, the two portions of the DFP were usually distinguishable on the basis of size. Whenever this



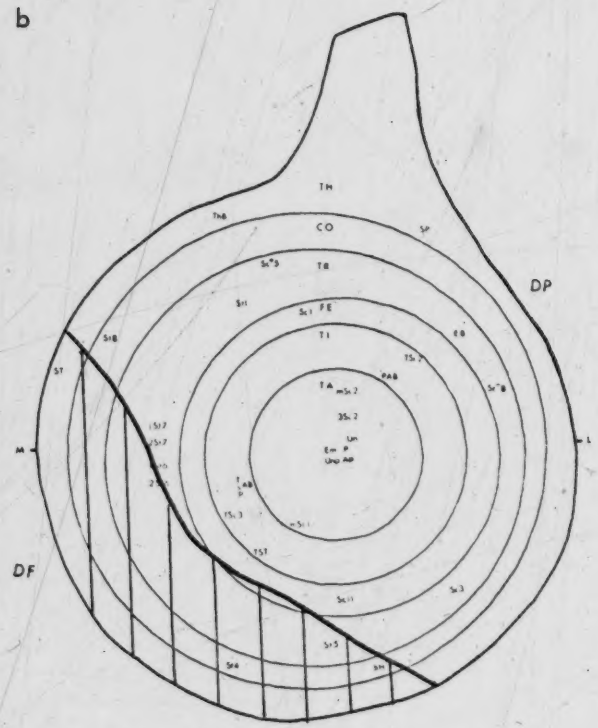


Figure 9 (a) An example of the legs found in a 726 female after a 29° treatment. One leg (the right leg) is morphologically normal. The left leg is a DFP. The marker designations are given in Table 2.

(b) The markers missing (DF) and present twice (DP) in the DFP shown in (a), shown on a fate map.

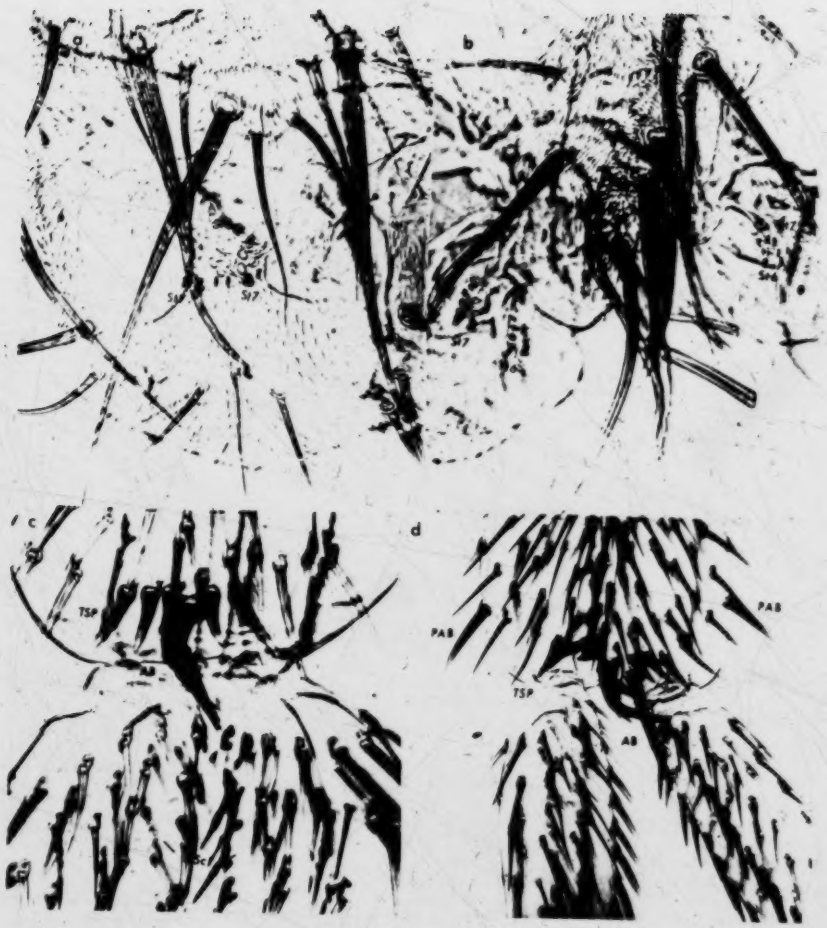


Figure 10 Examples of marker duplications found in DFP legs:
(a) trochanter with 1st7, 2st7 and 1st6 duplicated and 2st6 deficient
(b) trochanter with all rows of 2GST duplicated
(c) duplication with one AB and partially duplicated Tsp
(d) duplication with 2 AB and 2 sets of TSP.
Markers as in text (p.)

was the case, the duplicate portion was found to be smaller than the original. In some cases this difference was extreme. In the example shown in Figure 11, over half of the markers present in the original are not present in the duplicate. This is illustrated by the fate-map shown in Figure 11b, in which the areas not present in the duplicate are labelled "incomplete". Those duplicates not containing all of the morphological markers present in the original are referred to as *incomplete* duplicates. The source of this variation in the number of structures present in the duplicates is examined later.

Investigation of the DFP Phenotype

Several variables were considered in the investigation of the DFP phenotype. The frequencies with which each marker was deficient and duplicated, the size and location of the deficient and duplicated regions in the DFP's and the possible correlation of marker states within individual legs were examined. These variables were examined to determine whether a pattern of deficiency/duplication exists within the DFP's, and if so whether this pattern is the same in DFP's induced by 29° treatments given at different stages of development.

The data were from a sample of 893 DFP's from the 726 males in batches showing a high DFP frequency (34-90 hour heat treatment) in the experiment described above. Each DFP was scored under the compound microscope for the presence and number of elements in each of the 32 mesothoracic leg markers. The duplicate and original portions of each DFP were scored separately. The markers in each portion were recorded as a string of digits and entered onto computer cards for analysis. A separate computer card was used for the data from each portion of each DFP.

The layout of these computer cards is shown in Appendix B. Each

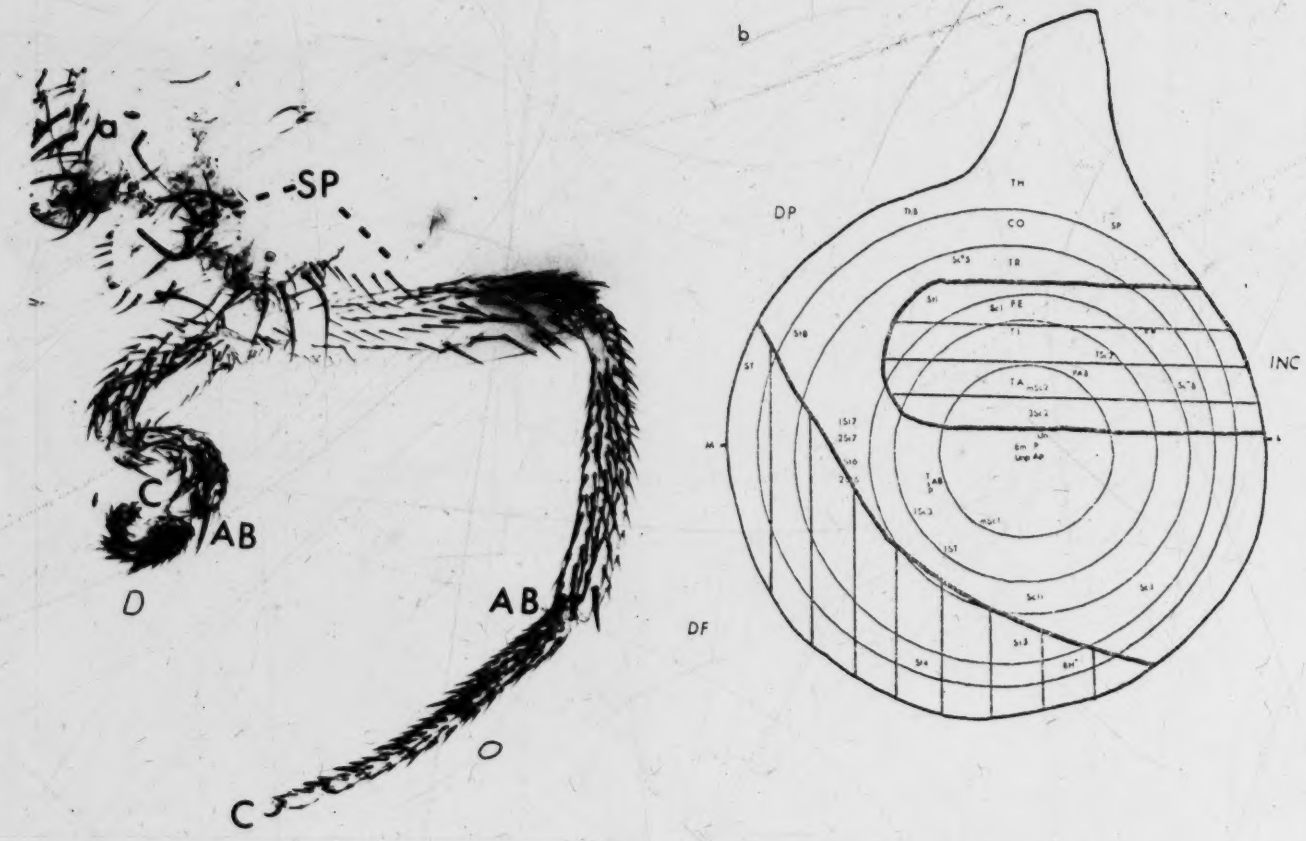


Figure 11 (a) An example of an incomplete duplicate. Some of the markers present in the original portion (O) are not present in the duplicate (D). The marker designations are given in Table 2.

(b) The markers missing (DF), present only in the original (INC) and present in both the original and duplicate (DP) in the DFP shown in (a), shown on a fate map.

card contained an identification number, indicating which treatment batch, fly and portion of DFP that set of data came from. The number of elements in each marker were recorded in a specific column or columns. This organization allowed computer subroutines to be used to sort and tabulate the data.

The first such computer subroutine was designed to recode the data. Each morphological marker in the normal leg contains a characteristic number of elements, as shown previously (Table 4). Thus in a DFP whether a marker is duplicated or deficient can be determined by comparison of the observed number of elements with the normal number of elements for that marker. For example, an $Sc^{+}5$ containing two sensilla is partially deficient while a mScl with two sensilla is duplicated. To facilitate comparison between markers the data were coded to indicate the status of each marker with respect to its normal state. The two cards containing the data from each DFP were retrieved and each marker was assigned a single numerical score which indicated whether it was deficient, duplicated or normal.

The recoding scheme used is illustrated in Table 6 which also shows the combinations of marker states actually encountered. If no elements were present a marker was coded as being deficient (1). If elements were present in only the original portion of the DFP the marker was coded as being not duplicated (2, 3, 4) as shown in Table 7. If some elements were present in both portions of the DFP the marker was coded as being duplicated (5). If more than two complete sets of markers were present the marker was coded as being triplicated (6).

This coding scheme can properly code as duplicated those cases in which less than one complete set of elements is present in both portions

Table 6 The Combinations of Marker States Observed in Original and Duplicate Portions of DFPs and the Codes [1] Assigned to each Combination

		Number of Elements Present in the Duplicate[2]			
		0	<Normal	Normal	>Normal
Number of Elements Present in the Original	0	1	-	-	-
	<Normal	2	5	5	5
	Normal	3	5	5	6
	>Normal	4	5	6	6

1. 1=Marker deficient
   2=Partial deficiency
   3=Normal state
   4=Increased number of elements
   5=Pattern duplication
   6=Triplication (not observed in this sample of DFPs)
   -=Combination of marker states not found in DFPs

2. The number of elements was considered normal when within two standard deviations of the mean in the 22° control leg sample. The normal range for each marker is shown in Table 7

Table 7 The Number of Elements Present in the Recoding Classes for Each Marker[1]

Marker	Number of Elements Present				
ST	0		1	2	3
ThB	0	1-4	5-7	8-14	15
SP	0	1	2	3-4	5
StB	0	1-7	8	9-16	17
BH	0		1	2	3
St4	0	1-3	4	5-8	9
1St7	0	1-2	3	4-6	7
2St7	0	1-3	4	5-8	9
1St6	0	1-2	3	4-6	7
2St6	0	1-2	3	4-6	7
St1	0		1	2	3
Sc 5	0	1-4	5	6-9	10
EB	0		1	2	3
Sc 8	0	1-7	8	9-16	17
Sc3	0	1-2	3	4-6	7
St5	0	1-4	5	6-10	11
Sc1	0		1	2	3
Sc11	0	1-10	11-12	13-23	24
TST	0	1-2	3-5	6-10	11
TSc2	0	1	2	3-4	5
TSc3	0	1-2	3	4-6	7
PAB	0		1	2	3
AB	0		1	2	3
Tsp	0	1-3	4-6	7-12	13
mSc1	0		1	2	3
mSc2	0	1	2	3-4	5
3Sc2	0	1	2	3-4	5
Un	0	1	2	3-4	5
Em	0		1	2	3
P	0	1	2	3-4	5
Unp	0		1	2	3
Ap	0		1	2	3
Recode value[1]	1	2	3	4	6

1. Recoded values: 1=Deficient; 2-Partial Deficiency; 3=Normal; 4=Increased number of elements; 6=Triplication (see text). Any marker with two or more elements present may be classified as 5, a pattern duplication (See text).

of the DFP. Such cases might well arise in incomplete duplicates or when only part of a marker is removed by the deficiency and the remaining part duplicates.

The Duplication and Deficiency Frequencies of Individual Markers

It was observed during the DFP scoring that the markers were not deficient and duplicated at random. Some markers were deficient often and duplicated rarely, while others were deficient rarely and often duplicated. To clarify this observation the number of cases in which each marker was given a score of 1 (deficient), 2-4 (not duplicated) or 5 (duplicated) were determined for the 893 DFP's scored. These numbers are given in Table 8, ordered according to the procedure explained below.

It is obvious that there is a great deal of variation in the frequency with which each marker is deficient. For example, the ST was deficient in 93% of the cases while over half of the markers were deficient in less than 1% of the cases. The frequency with which each marker is duplicated does not show such a wide fluctuation. Also, the fluctuation in duplication frequency was not obviously correlated with the fluctuation in deficiency frequency.

A question which arises at this point is the following: are there patterns in the variation in deficiency and duplication frequencies? One possibility is that these frequencies are correlated with the marker's position in the imaginal disc. Accordingly, the DF and DP frequencies were plotted on fate-maps (Figures 12 and 13). As can be seen from the figures, the average deficiency frequencies appear to be correlated with marker position. The deficiency frequencies are high medially and decrease laterally. The average duplication frequencies

Table 8 The Number of Deficient, Duplicated, and Normal Cases for Each Marker [1]

Marker	The Number of Cases Total[3]	DF	DP	N	DF/(Total)	DP/(Total)	DF/DP	Rank [2]
ST	872	813	34	25	.93	.039	23.91	1
2St6	889	470	285	134	.53	.32	1.65	2
St4	886	421	376	89	.48	.42	1.12	3
BH	885	408	404	73	.46	.46	1.01	4
1St6	889	314	362	213	.35	.41	.87	5
St5	889	272	390	227	.31	.44	.70	6
Sc11	883	138	592	153	.16	.67	.23	7
Sc3	890	124	595	171	.14	.67	.21	8
2St7	889	123	594	172	.14	.67	.21	9
St8	877	25	239	613	.029	.27	.10	10
1St7	889	19	776	94	.021	.87	.024	11
TSc3	876	16	695	165	.018	.79	.024	12
TST	875	15	701	159	.017	.80	.021	13
EB	887	8	441	438	.0090	.50	.018	14
AB	868	10	622	236	.012	.72	.016	15
3Sc2	732	3	239	490	.0041	.33	.012	16
St1	888	6	520	362	.0068	.59	.011	17
mSc1	760	5	513	242	.0066	.68	.0097	18
mSc2	753	4	419	330	.0053	.56	.0095	19
Sc1	881	5	530	346	.0057	.60	.0094	20
PAB	868	4	433	431	.0046	.50	.0092	21
TSc2	875	4	470	401	.0046	.54	.0085	22
TSP	868	5	702	161	.0058	.81	.0071	23
Sc+5	887	5	703	179	.0056	.79	.0071	24
Sc8	888	3	653	232	.0034	.74	.0046	25
Un	833	1	365	467	.0012	.44	.0027	26
ThB	724	1	390	333	.0014	.54	.0026	27
AP	836	1	408	427	.0012	.49	.0025	28
Unp	836	1	512	323	.0012	.61	.0020	29
Em	835	1	514	320	.0012	.62	.0019	30
P	835	1	532	302	.0012	.64	.0019	31
Sp	745	1	613	131	.0013	.82	.0016	32

1. In this analysis, DF=1 N=2,3,4 Dp=5. No cases of triplication (6) were observed.
2. The markers were ordered according to the DF/DP ratio, as described in the text.
3. Variation in number is due to physical damage during preparation.

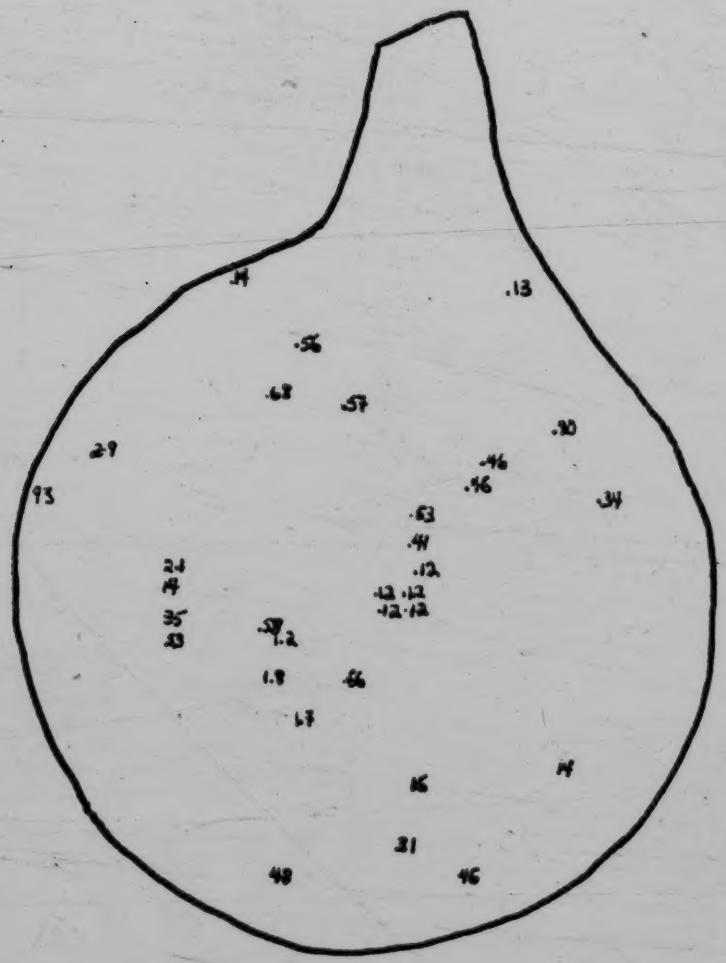


Figure 12 The frequencies with which the 32 mesothoracic leg markers were deficient in a sample of 893 DFPs, shown on a fate map (given as percentages).

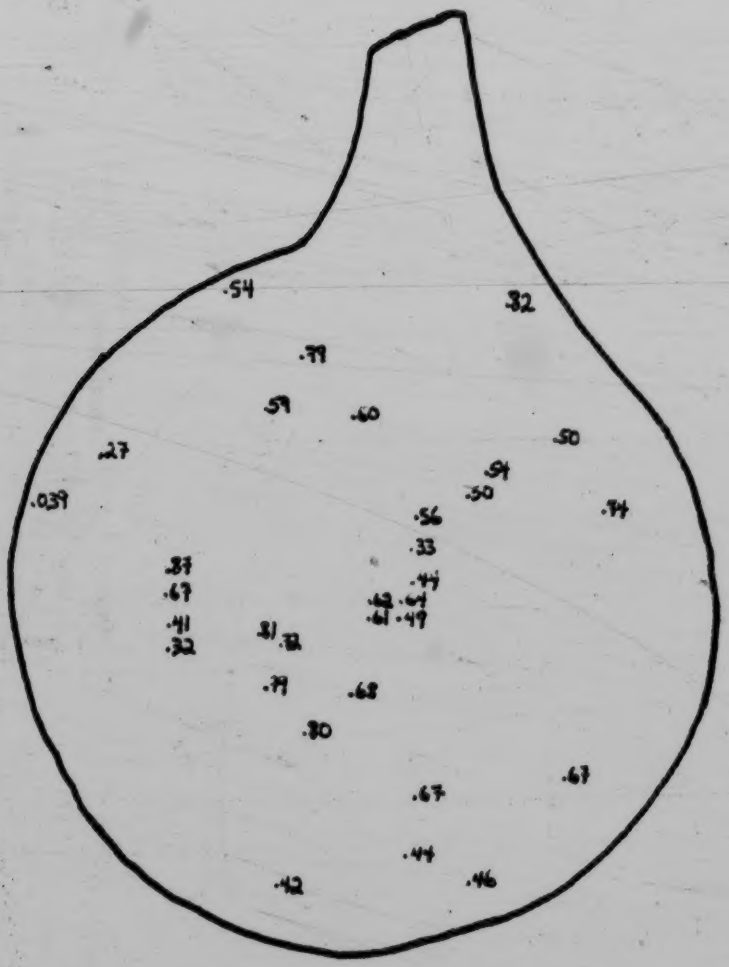


Figure 13 The frequencies with which the 32 mesothoracic markers were duplicated in a sample of 893 DFPs, shown on a fate map.

are not obviously correlated with marker position.

The ratio of the number of cases in which a marker is deficient to the number in which it is duplicated (DF/DP) was calculated for each marker to provide a convenient, unified measure of the variation between markers. The markers were then ranked by the value of this ratio, as shown in Table 8. The rank number of each marker was plotted on a fate-map (Figure 14). The markers in the upper medial quarter of the disc had the highest DF/DP values and the values descended laterally in a smooth gradient. The series of contours drawn on the fate-map illustrate this trend.

Caution must be used in interpreting this result as the method used to recode the DFP markers may influence the ranking. Those markers consisting of a single element (SC1, EB, etc) will have only a few precursor cells while those containing many elements (Sc11, Tsp, etc) will have many more. This means that in cases where the marker is close to, or touched by the boundary of the deficiency the small markers may be more often completely deficient than the large markers, which will be partially deficient and partially duplicated and hence classified as duplicated. This will be especially important when the precursor cells for single element markers are physically close to those of multiple element markers.

Given this caveat and given the low number of deficiencies observed for some of the more lateral markers, little confidence can be placed in the rank order of the markers in the lower portion of the table. Still, the pattern of the markers does not show any serious internal inconsistancies. Only two of the 32 markers do not easily fit within the series of simple contours drawn on the fate-map (Figure 14).

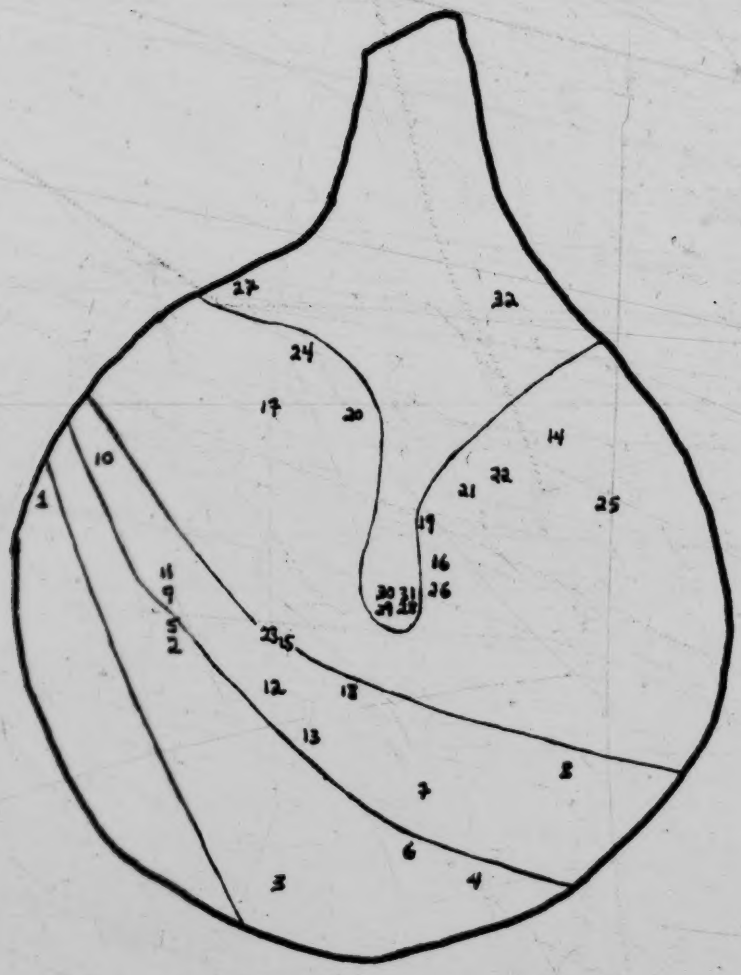


Figure 14 The DF/DP rank orders for each of the 32 mesothoracic leg markers shown on a fate map. The contour lines indicate the shape of the gradient.

The Location and Size of Deficiencies and Duplications in DFP's

The previous results indicate that medial markers are more often deficient than others in DFP's. However, the observed gradient in average DF/DP frequencies only gives an incomplete picture of the pattern of deficiency and duplication found in individual DFP's. A question which therefore arises is the following: are the sizes and locations of the deficiencies and duplications in individual DFP's responsible for the observed average gradient pattern.

To investigate this, the 893 DFP's were first classified according to the markers deficient. All DFP's with the same markers deficient were grouped together into a deficiency class. A total of 115 such deficiency classes were found in the sample of DFP's. The markers deficient in each class, and the number of DFP's in each class are given in Appendix C. A representative sample of the deficiencies was selected and the area deficient in each was traced on a fate-map. The resulting map is shown in Figure 15.

Several observations can be made from these results. First, the deficiencies generally affect contiguous sets of markers, i.e., the markers deficient in each case could usually be separated from the duplicated and normal markers on a fate-map by a single line. Secondly, the markers deficient were always those medial to this line. Thirdly, the deficiencies were usually at least partially overlapping, eg., while the borders of the deficiencies varied greatly, 831 of the 834 deficiencies were all deficient for ST, 449 were all deficient for ST and 2St6, 432 for ST, 2St6 and St4 and 343 for ST, 2St6, St4 and BH^-. These are the first, second, third and fourth most commonly deficient markers respectively. Thus the observed pattern of marker deficiencies appears

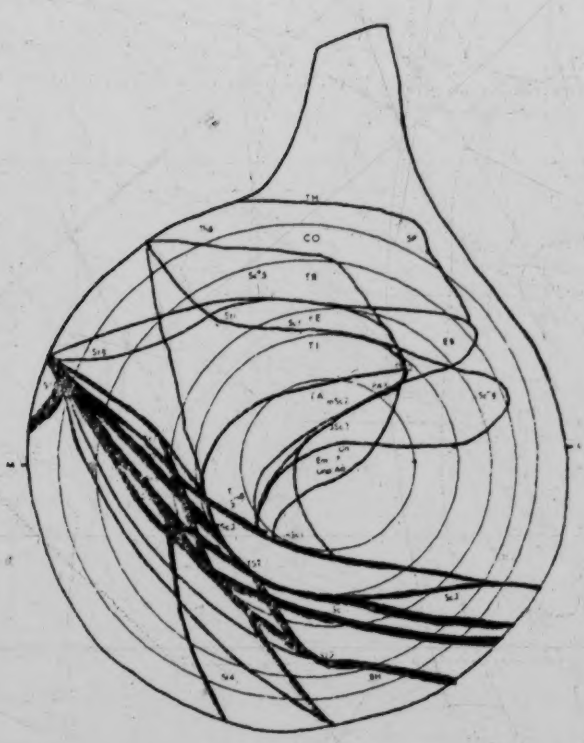


Figure 15 A diagram showing the boundaries of the area deficient in a representative sample of 170 DFPs, traced on a fate map. The area medial to the line was deficient in each case. The sample was chosen by taking examples from each of the 115 unique deficiency classes (Appendix C) according to the following scheme:

1-10 DFPs in the deficiency class = 1 example
11-20 DFPs in the deficiency class = 2 examples
21-30 DFPs in the deficiency class = 3 examples
31-40 DFPs in the deficiency class = 4 examples
41-50 DFPs in the deficiency class = 5 examples
51 or more DFPs in the deficiency class = 6 examples

to be the result of the deficiencies all being variants of a single category. They all affect medial, contiguous, partially overlapping sets of markers.

Fourthly, the number of markers deficient in the DFP's is variable, ranging from a single marker (ST) to 20 of the 32 markers scored (see Appendix C). Most of the deficiencies were small, for example, 303 DFP's were only missing the ST. At the extreme end of this range of deficiency size we may include another class of DFP's in which no markers were deficient. In view of the variation in deficiency size it seems logical to classify these on the basis of their duplications alone.

Finally, no indication of a difference in deficiency size between DFP's resulting from an early as opposed to a later 29° treatment was observed. The more frequent deficiency classes contained cases from all treatment batches, reinforcing the conclusion that all of the deficiencies are variants of a single category.

The pattern of duplication in the DFP's is not as clear as that of the deficiencies. The duplications vary from complete to only a few markers duplicated. A sample of cases with different duplicate completeness was selected and the areas deficient, normal and duplicated in each are shown in Figure 16, drawn on fate-maps. As can be seen, structures are not duplicated at random, but often appear to form a complete circle of markers in the disc. In more complete duplicates this circle is larger, until in nearly complete duplicates only one or two markers in the duplicate are in a different state from the original.

One possible explanation for this variation in completeness is that the 726 males are pupariating before the duplicate has completed development, since the 29° treatment cause a developmental delay of variable

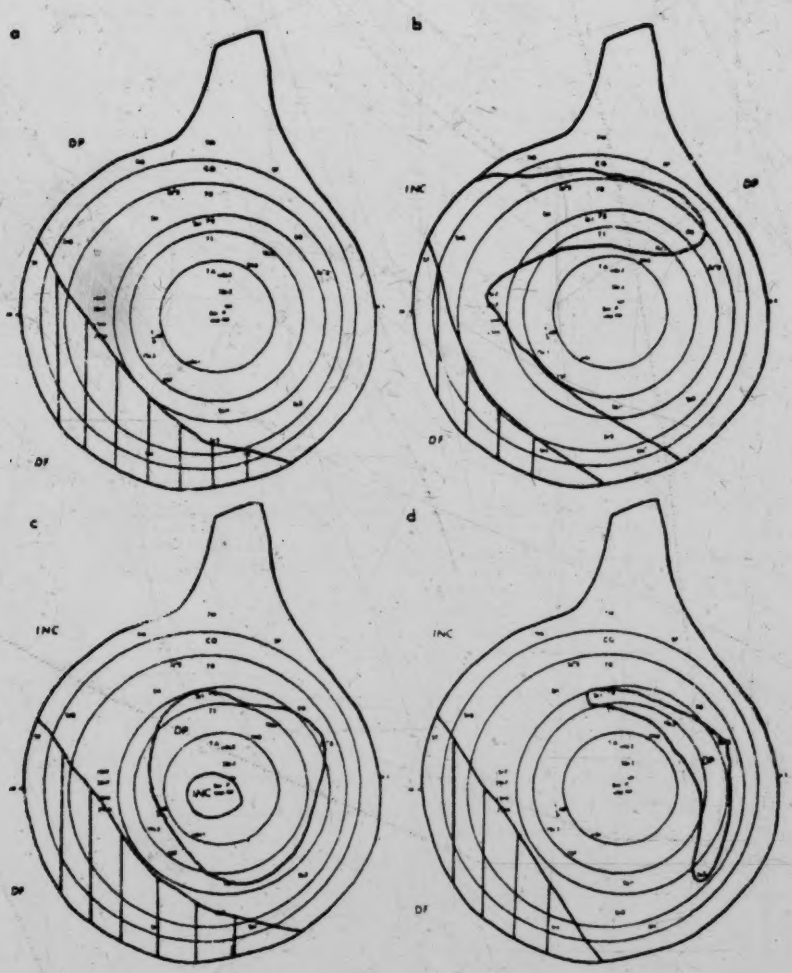


Figure 16 Fate map diagrams showing the areas deficient (DF), not duplicated (INC) and duplicated (DP) in four arbitrarily chosen DFPs.

duration (Russell 1974). This was tested by measuring the strength of the correlation between the duration of development of the 29° treated 726 males and the degree of incompleteness of the duplicates. The duration of development was measured as the total number of days between egg laying and eclosion. Incompleteness was measured as the ratio of the number of markers in the DFP with a recoded value of 2, 3, or 4 (i.e., not deficient and not duplicated) to the total number of markers present in the original. This ratio will vary between 0 and 1. If the 726 males with the latest eclosion date have more complete duplications this correlation is expected to be negative. The correlation coefficient, r, is -0.22. This is negative and significant ($P<.05$) however, r^2, the amount of the variation in the incompleteness that can be explained by the correlation is only .05. Thus only a minor fraction of the variation in the completeness of the duplicates can be attributed to individual differences in development time. This may be on account of the fact that many discs are undergoing regeneration simultaneously following the 29° treatment. This will weaken any relationship that might exist between the completeness of the leg duplication and pupariation.

Investigation of the Deficiency/Duplication Relationships in Individual DFP's

In the previous results the deficiency/duplication ratios for individual markers were found to form a gradient on the fate-map, but no such simple, average pattern could be found for duplications. To attempt to clarify this situation, individual DFP's were examined for evidence of a correlation between the states of neighboring markers.

A model has been proposed by Bryant (1971) that may be used to explain this behavior. He suggests that individual imaginal disc cells

have a fixed position in a gradient of developmental capacity and that cells are capable of regenerating only those structures with values lower in this gradient than their own. Thus a duplication would result from the removal of the high point of the gradient followed by growth of some of the remaining cells at the "cut edge" of the fragment. This growth would produce another copy of all of the structures lower in the gradient than the cells at the cut edge, leading to a duplication. The similar phenotype of duplications induced by *ts726* and by culturing disc fragments implies that the *ts726* discs in which a patch of cell death is induced are similar to disc fragments produced by surgery.

If this is the situation in the *ts726* system, then the local gradient levels of neighboring markers might be reflected in the deficiency/duplication pattern of each DFP. For each pair of neighboring markers the higher marker should be deficient and the lower duplicated, but never the reverse. The existence of only one type of deficiency/duplication pattern can thus be taken as an indication of the polarity relationship between two neighboring markers, with the deficient marker being higher in the gradient than the duplicated one.

The data from the 893 DFP's were analyzed for such polarity relationships. For each marker a set of "nearest neighbors" was chosen. These represented the closest surrounding set of markers on the fate-map. A computer subroutine was then used to scan each marker and its neighbor set in each DFP for cases in which the chosen marker was duplicated and only one of the markers in its neighbor set was deficient. Such a case was taken as a valid indication of a pairwise polarity relationship. DFP's in which two or more of the neighboring markers were deficient were eliminated, as the polarity relationships are not

clear in such cases. For each marker the number of cases indicating a polarity relationship with each of its neighbors was counted.

The results of this analysis are given in Table 9. Marker comparisons which yielded no valid cases are not shown. To test whether variation in the neighbor set used had an effect on the resulting relationships the analysis was run several times with smaller, less restrictive neighbor sets for each marker. The relationships shown in Table 9 were not materially affected by changing the neighbor set. The individual relationships indicated by the original test are shown in Figure 17, drawn on a fate-map. A double headed arrow is used to indicate those cases where some ambiguity exists as to the relationship. For example, in the BH^-:St5 comparison 6 cases indicate BH^- is higher than St5 and 3 cases indicate the opposite. However most comparisons yielded an unambiguous polarity as is shown. The overall pattern of relationships indicates that polarity is constant throughout the disc, as if a simple gradient exists with ST being at or near the high point and with values descending laterally. No internal inconsistancies exist in this pattern, i.e., paths formed by any combination of the arrows do not form closed circles.

This result agrees nicely with the expectations of the gradient model. According to this model each DFP is the result of regeneration in response to cell death removing the high point of a single gradient in the disc. If this were the case, the gradient high point would be located at or near the medial edge of the disc, very close to the marker ST.

The single gradient model has recently been superceded by a model involving two positional coordinates, an angular and a radial value (French et al. 1976). This model can account for all of the results

Table 9 The Polarity Relationships Indicated between Markers by the Nearest Neighbor Analysis

Markers A	B	Cases A>B	B>A	Markers A	B	Cases A>B	B>A	Markers A	B	Cases A>B	B>A
ST	ThB	503	0	BH^-	Sc8	191	0	2St6	Sc11	21	0
ST	St4	286	0	BH^-	Sc11	7	1	Sc1	TSc2	4	1
ST	St8	247	0	St5	Sc3	7	0	Sc1	PAB	1	0
ST	1St7	259	0	Sc11	St5	1	0	2St7	Sc1	79	0
ST	2St7	250	0	St5	TST	108	0	mSc1	TST	1	0
ST	1St6	266	0	2St6	St5	24	0	PAB	TSc2	1	0
ST	2St6	221	1	Sc3	Sc^-8	5	0	TSc2	mSc2	1	0
EB	SP	4	0	Sc11	Sc3	1	0	TSc3	mSc1	3	1
Sc5	SP	1	0	St1	Sc^+5	1	0	PAB	3Sc2	1	0
ThB	EB	1	0	Sc1	St1	4	1	AB	mSc1	1	1
St4	St8	1	0	St1	TSc2	1	0	AB	Un	3	0
St4		28	0	1St7	St1	66	0	AB	Ap	3	0
St4	St5	16	0	EB	Sc5	1	1	2St6	AB	110	0
St4	Sc3	34	0	Sc1	EB	2	1	2St6	Tsp	122	0
St4	Sc11	16	0	EB	TSc2	1	1	mSc1	Em	2	0
St4	1St6	1	0	EB	PAB	1	1	mSc1	P	3	0
2St6	St4	3	1	Sc1	Sc^5	4	0	mSc1	Unp	3	0
St8	St1	7	0	1St7	Sc^5	78	0	3Sc2	Em	1	0
St8	1St7	1	0	Sc8	Sc1	1	0	3Sc2	P	1	0
St8	2St7	1	0	Sc11	TST	9	0	2St6	1St7	6	0
2St6	St8	3	0	Sc11	TSc3	73	0	2St6	2St7	6	0
BH^-	St5	6	3	Sc11	AB	1	0	2St6	1St6	3	0
BH^-	Sc3	17	0	Sc11	Tsp	1	0				

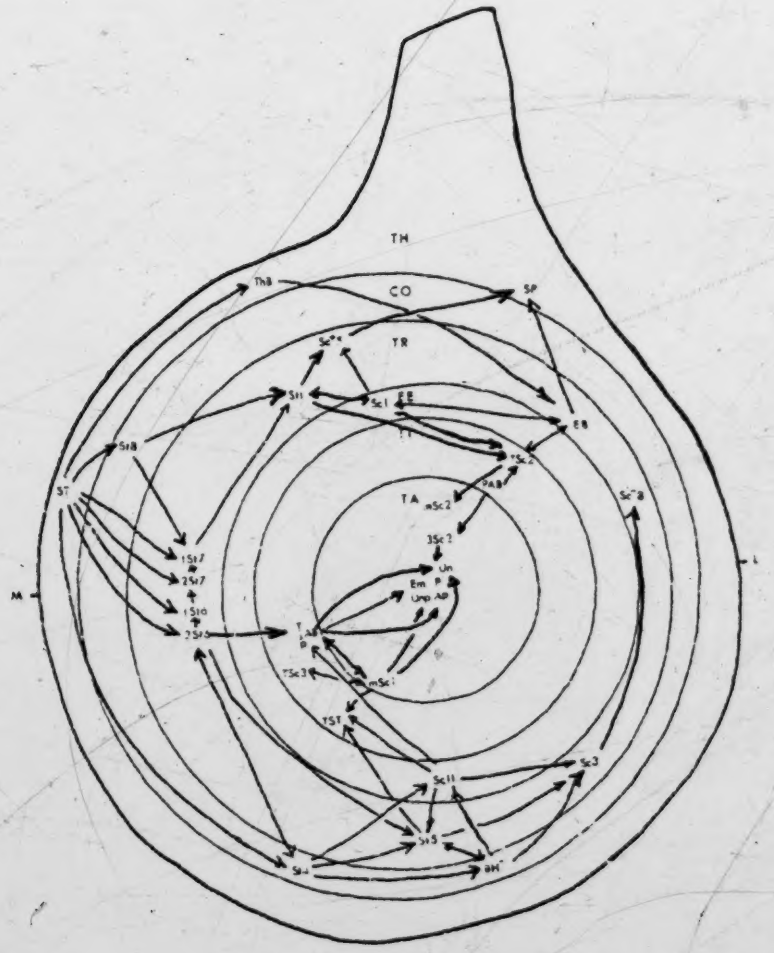


Figure 17 A fate map diagram showing the local polarity relationships indicated by the nearest neighbor analysis. A double headed arrow indicates an ambiguous result in which the tests gave conflicting results. Only those cases in which every example indicated the same polarity relationship between markers are shown with a single headed arrow.

explained by the original single gradient model and for additional data with which the earlier model cannot cope. In addition, this model can be used to make several predictions regarding the generation of a duplication. One of particular interest to the present work concerns the number of positional values of the leg disc which must be removed to initiate a duplication. The model predicts that half of the angular values of the disc must be removed for duplication to occur. The results of recent work (Schubiger 1971, Strub 1977 a,b) indicate the upper medial quarter of the first leg disc can regenerate all of the leg structures. This has been taken to mean that half of the positional values must be contained in the upper medial 1/4 or less of the first leg disc.

The data presented above bears on this conclusion. In many of the *ts726* induced DFP's only a small portion of the disc is deficient. For example, in 303 of the 893 DFP's only a single marker, the ST was deficient and in an additional 56 DFP's no marker was deficient. These data imply that the first and second leg might have a different organization of positional coordinates, that 1/2 of the angular coordinate values in the leg are compressed into only a very small portion of the upper medial quarter of the disc or that the model is incorrect.

However, there is an important difference in the techniques used to generate the duplications presented above and those on which the model is based. The model is based on the results of surgical fragmentation experiments in which cells are removed and the regenerative ability of the remaining fragment is observed. In the *ts726* system patches of cells were simply killed and their remains left *in situ* (Clark and Russell 1977). This difference, a clean cut vs. a patch of dead cells may be important in wound healing and in the interaction of the surviving cells of the disc, and thus in the initiation of a duplication.

In Vivo Culturing of Imaginal Disc Fragments

To test this difference an experiment was designed in which mesothoracic leg discs with small, surgically created deficiencies covering the same area as the small *ts726* induced deficiencies were cultured *in vivo*. The same genetic system and general procedure was used as in the disc transplantation experiment designed to test the fate-map. Fragments were made by removing a small portion of the upper medial quarter of the leg disc with a hollow, blunt needle, as described in the materials and methods. The region of the disc removed by these cuts is shown in Figure 18 on a photograph of an imaginal disc. A series of the fragments were implanted into mature larval hosts to metamorphose, as before. A second series was first implanted into the abdomens of adult females for 5 days of *in vivo* culturing prior to being implanted into a mature larval host for metamorphosis. If the small, medial cuts are identical in effect to the small medial deficiencies induced by *ts726*, then these fragments should duplicate.

The number and frequency with which scorable vesicles are recovered are given in Table 10. The control series shows a success rate of 20.0%, similar to that of the fate-map test. The *in vivo* culture series shows a lower success rate, as might be expected for the more complicated procedure. The numbers of elements present in each of the recovered vesicles is given in Table 11.

These results are not numerous, and must be analyzed cautiously, being of a very preliminary nature. Several observations can be drawn from these results which can serve as the focus for further experiments. First, in the control series the ST is always missing, and the markers surrounding it are often missing. This indicates that the cuts are removing only markers in the medial portion of the disc. The exact location

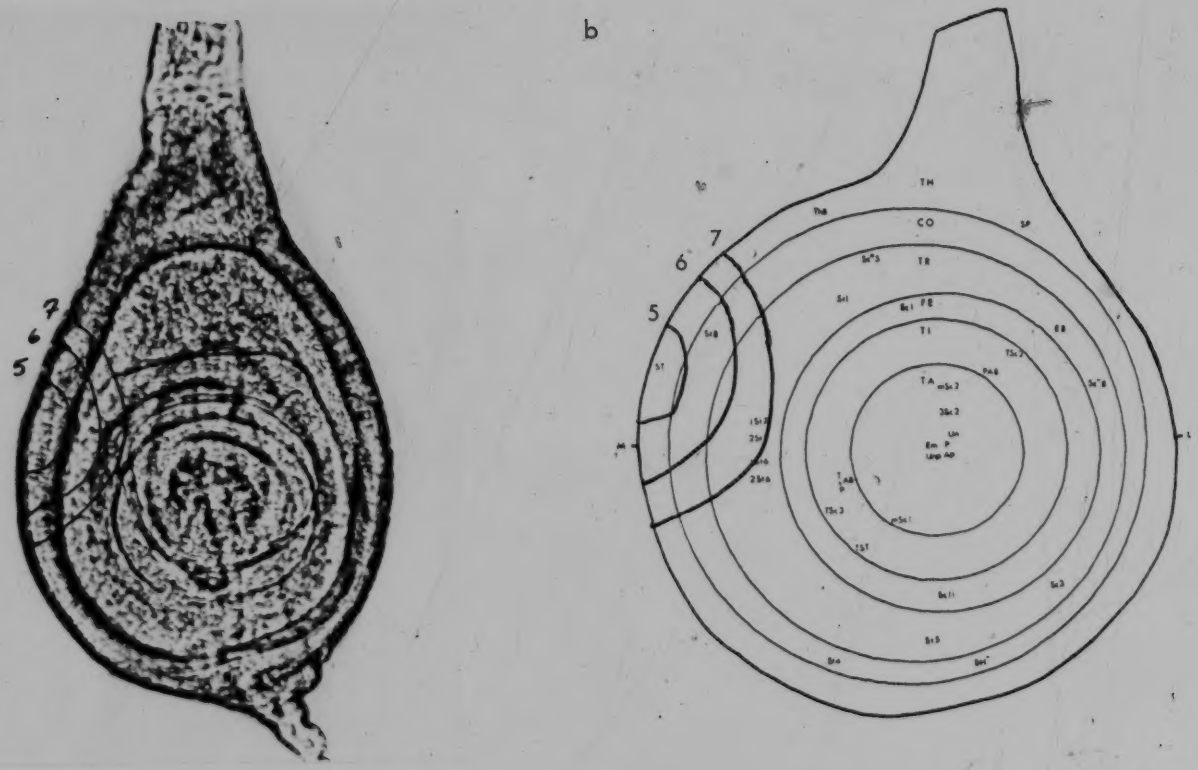


Figure 18 The area removed from the disc prior to culturing *in vivo* for 5 days. These cuts were made with a large hollow needle in the upper medial portion of the disc.
(a) The cuts on a photograph of the mesothoracic leg disc, and
(b) The cuts on a fate map of the disc. M = medial L = lateral

Table 10 The Frequency of Scorable Implants Recovered after 5 Days of *in vivo* Culture

Cut	Attempts	Scorable Implants Number	Percent
	controls		
5	10	3	
6	10	3	
7	10	0	
	30	6	20.0
	cultured implants		
5	35	5	
6	32	3	
7	20	1	
	87	9	10.3

Table 11 The Markers formed by Implants Cultured *in vivo* for 5 Days Prior to Metamorphosis

Implant Cut		ThB	Sc-5	St1	Sc1	St8	ST	1St7	2St7	1St6	2St6	TsP	AB	TSc3	TST	mSc1	St4	St5	Sc11	BH	Sc3	Sc 8	EB	TSc2	PAB	SP	mSc2	3Sc2	Ur.	P	Unp	Em	AP
5	1	6	5	1	1	8	1	3	4	3	3	5	1	3	3	1	4	5	11	1	3	8	1	2	1	2	2	2	2	2	1	1	1
	2	6	6	1	1	10	1	3	4	3	3	5	1	3	4	1	4	5	11	1	3	8	1	2	1	2	2	1	2	2	1	1	1
	3	5	5	0	0	8	1	3	4	3	3	5	1	2	3	1	4	5	11	1	3	8	1	2	1	2	2	1	2	2	1	1	1
	4	3	4	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	1	2	2	1	1	1
	5	4	5	1	1	10	1	3	4	3	3	5	1	2	3	1	4	5	11	1	3	8	1	2	1	2	2	2	2	2	1	1	1
6	1	6	5	1	1	10	1	3	4	3	3	5	1	3	4	1	4	5	11	1	3	8	1	2	1	2	2	2	2	2	1	1	1
	2	6	6	1	1	8	1	3	4	3	3	0	0	0	0	1	0	5	11	0	3	8	1	2	1	1	1	2	2	2	1	1	1
	3	0	0	0	0	0	0	0	0	0	0	5	1	3	4	0	0	0	0	0	0	0	0	2	1	1	2	2	2	2	1	1	1
7	1	4	5	1	0	0	0	0	0	0	0	5	1	2	4	1	0	0	0	0	0	8	1	2	0	1	1	2	2	2	1	1	1
Results of the control implants																																	
5	1	4	5	1	1	8	0	3	4	3	3	4	1	3	3	1	4	5	11	1	3	8	1	2	1	2	1	1	2	2	1	1	1
	2	3	5	1	1	4	0	0	0	0	3	3	1	3	3	0	4	5	10	0	3	8	1	2	1	2	2	2	2	2	1	1	1
	3	5	5	1	1	0	0	0	0	0	0	4	1	3	3	1	4	5	11	1	3	8	1	2	1	2	2	2	2	2	1	1	1
6	1	2	5	1	1	0	0	0	0	0	3	3	1	3	3	1	4	5	11	1	3	8	1	2	1	2	2	2	2	2	1	1	1
	2	3	0	0	0	0	0	0	0	0	0	3	1	3	3	1	4	5	11	1	3	8	1	2	1	2	1	1	2	2	1	1	1
	3	2	5	0	1	0	0	0	0	0	0	4	1	2	0	0	4	5	11	0	3	8	0	2	1	2	1	1	2	2	1	1	1
C	1	6	5	1	1	8	1	3	4	3	3	5	1	3	3	1	4	5	11	1	3	8	1	2	1	2	2	2	2	2	1	1	1
	2	5	5	1	1	8	1	3	4	3	3	5	1	3	3	1	4	5	11	1	3	8	1	2	1	0	0	0	0	0	0	0	0
	3	6	5	1	1	8	1	3	4	3	3	5	1	3	3	1	4	5	11	1	3	8	1	2	1	2	2	2	2	2	1	1	1
	4	6	5	1	1	8	1	3	4	3	3	5	1	2	2	0	4	5	11	1	3	8	1	2	1	2	1	1	2	2	1	1	1
	5	6	5	1	1	8	1	3	4	3	3	5	1	2	2	1	4	5	11	1	3	8	1	2	1	2	1	1	2	2	1	1	1

C=Control, uncut disc

of the deficiency varies from case to case, but it is usually smaller than the entire upper medial quarter of the disc. Secondly, no instances of pattern duplication were observed in the fragments cultured for 5 days. The markers present were only present once and usually with the normal number of elements. Finally, in 6 out of 8 cases the ST was present. An example of such a case is shown in Figure 19, along with a control vesicle. Although it is impossible, of course, to be certain of which structures were actually removed from these discs by the cut, the fact that all 6 of the controls were missing the ST implies that at least a majority of the cultured fragments were also missing the ST. Thus, these 6 cases are evidence for the regeneration of the ST.

As mentioned above, the preliminary nature of these results makes any conclusions tentative. The few cases which resulted from *in vivo* culturing do suggest that discs with small deficiencies created by surgically removing cells in the ST region do regenerate. This is in contrast to the results from the *ts726* system, suggesting that perhaps there may be a difference in the response of an imaginal disc to deficiencies induced by these two techniques. One possible explanation for this difference might be that dead cells interfere with the wound healing process in a manner which prevents regeneration and induces duplication.

One important fact which has not been determined is the exact size of a surgically induced deficiency required to induce a duplication. An attempt was made to induce duplications by culturing 1/2 disc fragments (the lateral fragments, 1B). Only one vesicle was recovered. In this a duplication of the tarsus had occurred. This is obviously an area where further work must be done before any definitive conclusions can be made.

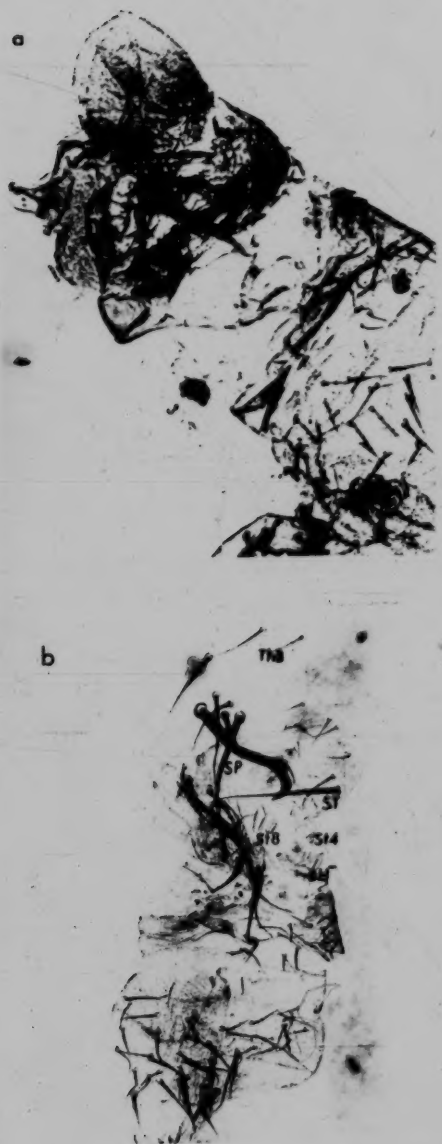


Figure 19 Photographs of the vesicles resulting from fragments generated by the cuts shown in Figure 18.
(a) when implanted into a mature larval host
(b) when cultured *in vivo* for 5 days prior to implanting into a mature larval host. The ST is present. The marker designations are given in Table 2.

The Effects of Variations in Treatment Conditions on DFP Frequency

This section of the results deals with the treatment conditions required for the induction of DFP's using the *ts726* system. Very few DFP's were induced in an early experiment set up to do a clonal analysis of the DFP's induced by *ts726*, and a number of tests were done to determine the cause of this low DFP frequency. The effects of varying the 29° treatment conditions, of combining 29° treatments with radiation treatments and of changes in the composition of the culture medium were tested.

Experimental Procedure

The experiments reported in this section were all done using the same genetic stocks and mating scheme. In each experiment a number of *Dp(3:1)mwh*$^{+}$, *mwh*$^{+}$ *y v f ts726/FM7a;mwh/mwh* virgin females were mated to *w sn*^{3} *ts726/B*^{s}*Y;mwh e/mwh e* males to generate *mwh*$^{+}$ *y v f ts726/ mwh*$^{+}$ *y v f ts726;mwh e/mwh* experimental females (called 726 females) and *w sn*^{3} *ts726/FM7a;mwh e/mwh* females (called FM7 females) that served as an internal viability control.

The Initial Clonal Analysis Experiment

In the initial experiment eggs from the mating were collected over 12 hour intervals and were divided into 10 batches for treatment. Three batches served as controls, one continuously incubated at 22°, one continuously incubated at 29° and the third given a 66 hour 22°/ 48 hour 29° treatment. The remaining 7 batches were given 66 hour 22°/ 48 hour 29° treatments and were irradiated at one of several times in development.

The 726 and FM7 females, both eclosed and pharate adults, from each batch were collected, counted and the 726 females were scored for DFP's

under the dissecting microscope as described in the materials and methods. The resulting DFP and survival frequencies for each batch are shown in Table 12. No 726 females survived in the 29° control and 100% 726 female relative survival with no DFP's occurred in the 22° control, as expected. The 29° treated, unirradiated control shows reasonable (24.6%) DFP and relative survival (37.3%) frequencies. These are somewhat different from the previous experiment, but some differences are to be expected considering that different genetic stocks were used.

Two highly unexpected results occurred in the 29° treated and irradiated batches. First, the batches irradiated between 54 and 210 hours show very low (0.3-5.3%) DFP frequencies and very high (99.3-100%) relative survival of 726 females. Secondly, the batch irradiated at 36 hours shows just the reverse, a high (57.1%) DFP frequency and a low (14.5%) survival. These results are quite different from the results of the morphological experiment given previously (Table 3).

Three differences between the conditions of this experiment and the previous *ts726* experiment were considered as possible causes of one or both of these unusual results. First, different genetic stocks were used. It is possible that a genetic modifier of *ts726* was present in these stocks which increased or otherwise changed the 29° treatment required to induce DFP's. This could account for the first result. Secondly, this experiment is the first in which combined radiation and 29° treatments were used. Irradiation is known to kill *Drosophila* imaginal disc cells (Haynie and Bryant 1976) and has been used to induce duplications when given to young larvae (Postlethwait and Schneiderman 1973, Postlethwait 1975). Thus it is possible that the second unusual result was caused by the irradiation of young 726 females. Thirdly, it was learned,

Table 12 The Results of the Initial Clonal Analysis Experiment

Larval Age at Irradiation[1] (hr)	Number of FM7 Females	Number of 726 Females	Relative[2] Survival of 726 Females (%)	DFPs Number	DFPs Percent[3]
36	482	70	14.5	80	57.1
54	358	443	> 100	47	5.3
114	1357	1433	> 100	12	0.4
138	1776	1764	99.3	21	0.6
162	1096	1135	> 100	9	0.4
186	1153	1343	> 100	6	0.2
210	599	662	> 100	10	0.8
Unirradiated Controls					
22°	1665	749	> 100	0	0
29°	1246	0	0	-	-
66/48[4]	480	179	37.3	88	24.6

1. Given in hours from the midpoint of the egg collection
2. Calculated as the number of 726 females/ the number of FM7 females
3. Percent of total legs
4. Indicates a 66 hour 22° /48 hour 29° treatment

after the fact, that some of the medium used in the initial experiment did not contain chloramphenicol (Dr. D. Nash pers. comm.). Since *ts726* was originally isolated on, and up to that time always cultured on medium containing chloramphenicol this difference might also have been important. Experiments were designed to test each of these possibilities.

The Response of the *ts726* Stocks to Varying 29° Treatment Conditions

This experiment was designed to determine the DFP and survival frequencies of 726 females resulting from different 29° treatments. If there is a modifier or some other factor affecting *ts726* in these stocks then only low frequencies of DFP's should result from a 66 hour 22°/48 hour 29° treatment, but, higher frequencies might result from a 29° treatment of longer than 48 hours or from a 29° treatment given at some other time in development. Accordingly, 8 batches of eggs were collected on medium containing 100 µg/ml chloramphenicol and were given different 22°/29° treatments. Two batches served as controls, one continuously incubated at 22° and the other continuously incubated at 29°. Three batches were given 29° treatments of varying length, and the remaining batches were given 48 hour 29° treatments at different times in development.

The DFP and relative survival frequencies for the 726 females in each batch are shown in Table 13. No 726 females survived in the 29° control, and 100% relative survival with no DFP's occurred in the 22° control, as expected. Increasing the length of the 29° treatment caused a decrease in the DFP frequency but had no appreciable effect on survival. The longest (66 hours) 29° treatment gave a lower (17.1%) DFP frequency than the 66 hour 22°/48 hour 29° treatment (32.1%). The timing of the 29° treatment did have an effect on both the DFP and the relative survival frequencies. The later treatment (90 hour 22°/48 hour 29°) had the

Table 13 The DFP Frequency in and the Relative Survival of *726* Females After Different 29° Treatments

Hours at 22°[1]	Hours at 29°	Numbers of FM7 Females	Numbers of *726* Females	Relative Survival of *726* Females (%)	DFPs #	DFPs %
72	66	192	38	19.8	13	17.1
72	60	240	50	20.8	29	29.0
72	54	212	40	18.9	24	30.0
90	48	333	7	2.1	2	14.3
78	48	133	71	53.4	33	23.2
66	48	46	14	30.4	9	32.1
Controls						
-	Constant	220	0	0	-	-
Constant	-	213	232	100	0	0

1. Before exposure to the 29° treatment

lowest (14.3%) DFP frequency and the lowest (2.1%) relative survival frequency. Relative survival was higher (53.2%) and DFP frequency intermediate (23.2%) following the 70 hour 22°/48 hour 29° treatment.

These results indicate that there is not a narrow range of 29° conditions for the production of DFP's using these stocks. Also, the 66 hour 22°/48 hour 29° treatment results in a reasonably high frequency of DFP's and a less than 100% relative survival of 726 females. The results agree well with the results of the unirradiated control batch in the initial experiment and indicate that the response of these stocks to 29° treatments has not been altered in such a manner as would explain either of the unusual results.

The Effects of Radiation Treatments on DFP Frequency and Survival

As noted previously, radiation treatments of young larvae have been used to induce duplications in *Drosophila* (Postlethwait and Schneiderman 1973, Postlethwait 1975). The frequencies produced in these experiments were low but it is nonetheless possible that the radiation treatment given at 36 hours was responsible for the high DFP frequency in the 36 hour batch. It is also possible that radiation later in development supresses DFP production, i.e., that the late irradiation was responsible for the low DFP frequencies in the 48-216 hour batches. To test these possibilities, two experiments were done to determine the effects of varying the dose and timing of radiation treatments, both in combination with 29° treatments and on 22° controls.

In the first experiment eggs were collected and divided into 19 batches for treatment. Three batches served as controls, one was continuously incubated at 22°, one was continuously incubated at 29° and the third was given a 66 hour 22°/48 hour 29° treatment without irradia-

tion. Eight of the batches were given the same 29° treatment and were also irradiated with different doses. Four were irradiated prior to and four following the 29° treatment to assess the effects of early vs. late irradiation. The final 8 batches were continuously incubated at 22° and were irradiated with the same doses at the same times as the 29° treated batches. The number of eggs in each batch varied. The 22° irradiated batches consisted of 6-10 bottles, each with about 125 eggs, a total of 800-1200 eggs. The rest consisted of the same, or were 2 to 3 times as large. This was done in an attempt to obtain a reasonable number of individuals from the treatments with low survival.

The DFP and relative survival frequencies for the 726 females in each of these batches are shown in Table 14. The three controls responded as before, with no 726 females at 29°, equal numbers of FM7 and 726 females and no DFP's at 22° and a reasonable DFP frequency and lower relative survival following the 29° treatment without irradiation. Three observations can be made from the results from the irradiated batches. First, early irradiation decreases the relative survival of, and induces some DFP's in 726 females at 22°. However, the effect is small at low (1000-2000 rads) doses. Considering the size of the dose given in the initial experiment (1500 rads), radiation alone cannot be responsible for the low DFP frequencies in the initial experiment. Secondly, irradiation at 36 hours followed by a 29° treatment produces a marked increase in DFP frequency and a reduction in the relative survival frequency. The DFP frequency following a combined treatment with 1000 or 2000 rads was greater than the sum of the DFP frequencies from the radiation treatment and the 29° treatment alone. This suggests that an interaction occurs between the effects of these two treatments. The

Table 14 The Effects of Early and Late Radiation Treatments on DFP and Survival Frequencies in *726* Females

29° Treatment (Hrs at 22°/ Hrs at 29°)	Time of Irr. (Hr)	Radiation Dose (Rads)	Number of FM7 Females	Number of *726* Females	Relative Survival of *726* Females (%)	DFPs in *726* Females #	DFPs in *726* Females %
72/48	-	0	426	288	67.6	171	29.7
72/48	36	1000	184	85	46.2	90	52.9
72/48	36	2000	76	5	6.6	6	60.0
72/48	36	3000	3	0	0	-	-
72/48	36	4000	0	0	0	-	-
72/48	168	1000	633	304	48.0	207	34.0
72/48	168	2000	296	116	39.2	56	24.1
72/48	168	3000	467	233	49.9	100	21.5
72/48	168	4000	207	85	41.1	27	15.9
22° Constant	-	0	290	289	100	0	0
"	36	1000	289	303	100	5	0.8
"	36	2000	203	101	49.8	38	18.8
"	36	3000	71	5	7.0	8	80.0
"	36	4000	0	0	-	-	-
22° Constant	168	1000	178	183	100	0	0
"	168	2000	222	248	100	0	0
"	168	3000	53	65	100	0	0
"	168	4000	9	6	66.7	0	0
29° Constant	-	0	662	0	0	-	-

magnitudes of the DFP and survival frequencies for the combined treatments are such as to suggest that this effect may be the cause of the high DFP frequency and low survival in the 36 hour irradiated batch in the initial experiment. Thirdly, no marked effect of late irradiation was observed, although higher doses of radiation decreased the number of FM7 and 726 females. No DFP's were induced by late irradiation at 22°, by even the highest dose given.

Thus the combination of a 29° treatment with a radiation treatment shows no effect which might explain the low DFP frequencies and high survival of the irradiated and 29° treated batches in the initial experiment. However, the effect of early irradiation followed by a 29° treatment may have been the cause of the high DFP frequency and low survival in the 36 hour irradiated batch.

This possibility was further investigated by an experiment which was designed to measure more carefully the effect on DFP frequency of irradiation at several times in development.

A sample of eggs was collected on normal medium and divided into 11 batches for treatment. Three batches served as controls, one continuously incubated at 22°, one continuously incubated at 29° and one given a 72 hour 22°/48 hour 29° treatment. Four of the batches were given a 72 hour 22°/48 hour 29° treatment and 1500 rads at different times in development. The final 4 batches were continuously incubated at 22° and were also given 1500 rads at different times in development.

No 726 females survived in the 29° control batch, and no DFP's were produced in the 22° control, as expected. The 29° treated, unirradiated control showed a reasonable DFP frequency, and the 24, 36 and 48 hour irradiated, 22° continuous batches showed a small DFP frequency (Table 15). The early irradiated, 29° treated batches show a

Table 15 Effect of combined 29° treatment and irradiation on DFP frequencies (%) in 726 females

	Not 29° treated	29° treated	X^2 for interaction component[1]	Prob.
Not irradiated	0.0	29.7		
Irradiated at 24 hrs	2.4	57.9	4.02	.05>P>.025
Irradiated at 36 hrs	1.0	56.5	3.51	.10>P>.05
Irradiated at 48 hrs	0.4	32.1	1.90	.25>P>.10
Irradiated at 168 hrs	0.0	28.7	0.00	1.00

[1] Determined from 2x2x2 X^2 analyses (Bishop, Fienberg and Holland '75) comparing in turn each irradiation with the unirradiated control.

greater than additive increase in the DFP frequency for irradiation given at 24 hours, but this effect decreased with increasing time of irradiation.

The significance of this interaction between the effects of the early irradiation and the 29° treatment was tested for each of the 4 irradiation times. This was done by arranging the data for each irradiation in a 2x2x2 contingency X^2 table (Bishop et al. 1975) with irradiated/not irradiated, 29° treated/22° constant and DFP/normal as the three variables. The 22° control and 29° treated unirradiated control data were used in each table.

In Table 15 the X^2 for the interaction component and the probability of its deviation from 0 by chance alone are given. At 24 hours the interaction is significant ($P = 0.03$) and at 36 hours it is marginally significant ($P = 0.06$). However, at 48 hours and 168 hours the interaction is not significant.

The combined treatment DFP frequencies shown here agree well with that observed in the 36 hour irradiated batch in the initial experiment (57.1%). Taken together with the previous results, this experiment provides convincing evidence that the second of the unusual results was due to an interaction between the effects of the radiation given and the 29° treatment. An important point to be considered is the time at which these DFP's are being induced. Since the irradiation alone produces few DFP's, it is likely that the majority are being induced at the time of the 29° treatment, i.e., the radiation is somehow increasing the sensitivity of imaginal disc cells to the effects of the 29° treatment.

These results do not, however, provide any explanation for the first of the unusual results, the low DFP frequency and high relative survival

of 726 females in the 54-210 hour irradiated groups in the initial experiment.

Effects of Chloramphenicol in the Culture Medium

It was discovered after the initial experiment that some of the media used had not been supplemented with chloramphenicol, as was routinely the case (see materials and methods). This change was investigated as a possible explanation for the first unusual result. First, the effects on the DFP and survival frequencies of varying the amount of chloramphenicol were tested. Twelve batches of eggs were collected and reared on medium containing differing amounts of chloramphenicol. One batch on chloramphenicol free medium was continuously incubated at 29° as a control. Four batches were given a 66 hour 22°/48 hour 29° treatment and the remaining 7 were continuously incubated at 22°.

The frequency of DFP's in and relative survival of the 726 females in each batch is shown in Table 16. No 726 females survived in the 29° continuous control, indicating that *ts726* is still a lethal at 29° on chloramphenicol free medium. The 22° continuous batches had no DFP's and 100% relative survival indicating that at 22° even at very high levels (up to 10 times that of normal medium), chloramphenicol alone does not induce DFP's. The 29° treated batches, however, had different DFP and survival frequencies, depending on the amount of chloramphenicol in the medium. The batch on chloramphenicol free medium had a low (5.9%) DFP frequency and 100% relative survival. The batch on 50 µg/ml (1/2 the normal amount) medium had an intermediate (12.5%) DFP frequency and 95.5% relative survival. And, the batch on normal medium had the usual (25.5%) DFP frequency and lower (71.0%) relative survival. Increasing the chloramphenicol to twice the normal amount did not further increase

Table 16 The DFP Frequencies and Relative Survival of *726* Females on Medium Containing Differing Amounts of Chloramphenicol

Treatment (Hrs at 22°/ Hrs at 29°)	Amount of Chloramphenicol in the medium (µg/ml)	Number of FM7 Females	Number of *726* Females	Relative Survival of *726* Females (%)	DFPs in *726* Females #	DFPs in *726* Females %
66/48	0	104	112	>100	12	5.9
66/48	50	67	64	95.5	16	12.5
66/48	100	69	49	71.0	25	25.5
66/48	200	60	42	70.0	21	26.2
22° Constant	0	93	109	>100	0	0
"	50	193	201	>100	0	0
"	100	225	248	>100	0	0
"	200	295	306	>100	0	0
"	400	260	282	>100	0	0
"	500	176	226	>100	0	0
"	1000	33	34	>100	0	0
29° Constant	0	76	0	0	-	-

the DFP frequency nor decrease the relative survival.

As a conclusive test of this effect, a second experiment was designed which generated a dose response curve for chloramphenicol. Fourteen batches of eggs were collected on medium containing different amounts of chloramphenicol. Two batches served as controls, one continuously incubated at 22° and the other continuously incubated at 29°, both on medium containing no chloramphenicol. The remaining 12 batches were given 72 hour 22°/48 hour 29° treatments.

The DFP frequency in and the relative survival of the 726 females in each batch are shown in Table 17, and the effect of the differing amounts of chloramphenicol is illustrated in Figure 20. No 726 females survived in the 29° control and 100% relative survival with no DFP's occurred in the 22° control, as expected. The DFP and survival frequencies in the remaining batches show a definite dependence on the level of chloramphenicol in the medium. At 50-70 µg/ml the DFP frequency begins to rise, reaching 25.5% at 100 µg/ml, the normal dose. The relative survival of 726 females remains at nearly 100% up to a dose of 70 µg/ml. At doses higher than this it declines, reaching 71.7% at 100 µg/ml. Again, increasing the dose to twice that of the normal medium did not appreciably effect the DFP or relative survival frequencies.

The results of these two experiments convincingly indicate that the first unusual result of the initial experiment, the low DFP frequencies in and the high relative survival of 726 females, were due to the lack of chloramphenicol in the medium. As a final test, the DFP and survival frequencies produced by combined 29° and irradiation treatments on chloramphenicol free medium were examined to determine whether the previously noted interaction is also dependent on the medium composition.

Table 17 The Chloramphenicol Dose Response Curve

29° Treatment (Hrs at 22°/ Hrs at 29°)	Amount of Chloramphenicol in the medium (μg/ml)	Number of FM7 Females	Number of 726 Females	Relative Survival of 726 Females (%)	Incidence of DFPs in 726 Females (#)	(%)
72/48	0	244	224	91.8	8	1.8
72/48	10	148	151	100	7	2.3
72/48	20	213	206	96.7	9	2.2
72/48	30	111	113	100	8	3.6
72/48	40	188	180	95.7	13	3.6
72/48	50	45	42	93.3	8	9.5
72/48	60	136	128	94.1	18	7.0
72/48	70	100	96	96.0	14	7.3
72/48	80	114	97	85.1	25	12.9
72/48	90	140	105	75.0	38	18.1
72/48	100	135	96	71.1	49	25.5
72/48	200	161	114	71.4	59	26.3
22° constant	0	169	174	100	0	0
29° constant	0	418	0	0	-	-

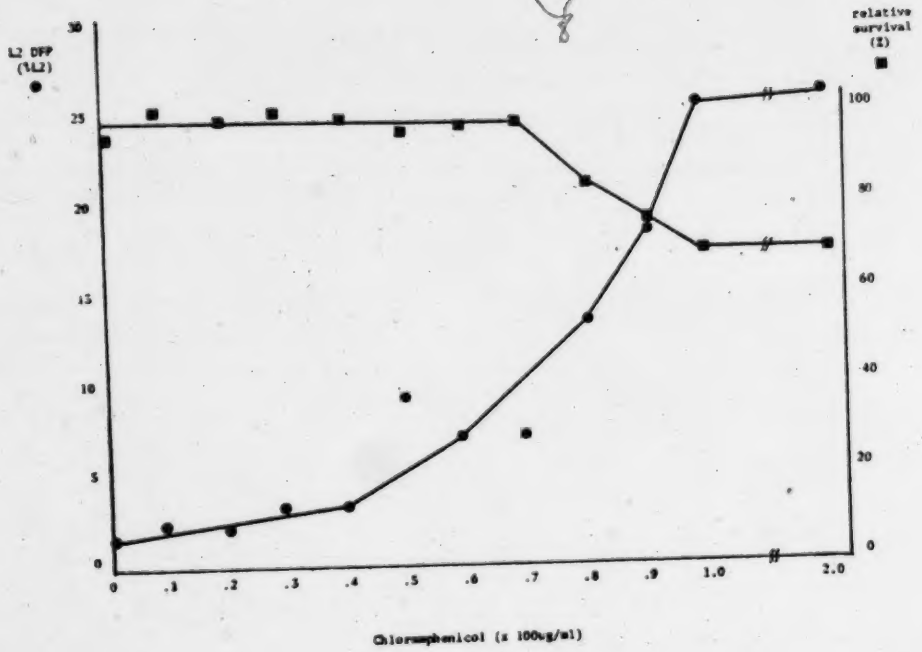


Figure 20 The dose response curve for chloramphenicol in the medium. The DFP frequency increases and the relative survival frequency decreases with increasing amounts of chloramphenicol.

In this test 8 batches of eggs were collected, either on normal medium or on medium without chloramphenicol. Three batches served as controls, one was continuously incubated at 22° and one was continuously incubated at 29° on medium containing no chloramphenicol. The third control was given a 66 hour 22°/48 hour 29° treatment on normal medium. Three additional batches were given 66 hour 22°/48 hour 29° treatments on medium containing no chloramphenicol, two of which were irradiated with 1500 rads. The remaining two batches were continuously incubated at 22° on chloramphenicol free medium and were also irradiated.

The DFP and survival frequencies of the 726 females in each batch are given in Table 18. The 22° and 29° controls are as expected. The 29° treated control shows an appreciable DFP frequency (27.0%) and a lower relative survival (61.5%). The 29° treated and early irradiated batch shows a DFP frequency of 48.8%, which is much higher than the sum of the two individual treatments (6.8%). Thus the synergistic interaction still occurs and produces a final DFP frequency of the same magnitude on chloramphenicol free medium.

Summary of the Results

Two interesting phenomena have been discovered in these experiments. First, the induction of significant numbers of DFP's in and the decrease in relative survival of 726 females by a 48 hour 29° treatment have been shown to be dependent on the presence of chloramphenicol in the culture medium, at nearly the normal dosage (100 µg/ml). Secondly, a synergistic interaction occurs between the effects of a 48 hour 29° treatment and a radiation treatment given early in development (24-48 hours) which leads to an increase in the DFP frequency in and a decrease in relative survival of 726 females.

Table 18 The Effects of Varying the Amount of Chloramphenicol in the Medium on the Radiation - 29° Treatment Interaction

29° Treatment (Hrs at 22°/ Hrs at 29°)	Amount of Chloramphenicol in the Medium (µg/ml)	Radiation Dose (rads)	Time[1]	Number of FM7 Females	Number of 726 Females	Relative Survival of the 726 Females (%)	DFPs in 726 females (#)	(%)
66/48	100	0	-	403	248	61.5	134	27.0
66/48	0	0	-	270	260	96.3	25	4.8
66/48	0	1500	36	66	41	62.1	40	48.8
66/48	0	1500	162	246	209	85.0	13	3.1
22° constant	0	0	-	276	269	97.2	0	0
22° constant	0	1500	36	181	198	100	8	2.0
22° constant	0	1500	162	123	117	90.2	0	0
29° constant	0	0	-	794	0	0	-	-

1. Given in hours after the midpoint of the egg collection period

In the initial experiments the most likely explanation for the reasonably high (24.6%) DFP and lower relative survival frequencies of the 29° treated, unirradiated control batch is that some of the medium used did contain chloramphenicol and that this batch was collected and reared on this medium. Caution must be used in comparing relative survival frequencies between experiments as survival is undoubtedly affected by many uncontrolled factors. For example, in the 29° treated control mentioned above, the relative survival was 37.3% where as in the later experiments survival on normal medium was usually around 70%. An increase in the experience of the experimenter may play a large factor in this difference between experiments.

Somatic Clonal Analyses

The third section of the results deals with clonal analysis of DFP's. Two analyses were done in which the frequency of clonal induction, the size of induced clones and the locations of clones were used to determine the number of cells present in and the patterns of growth of samples of DFP's and morphologically normal legs from the same flies. These results are compared with those from a series of 22° control legs. I was particularly interested in determining whether the intermediate development of duplicate portions of DFP's is similar to that of normally developing legs.

Experimental Procedure

A large number of *mwh*$^{+}$ *y v f ts726/w sn*^{3} *ts726;mwh/mwh e* females (726 females) and *w sn*^{3} *ts726/FM7;mwh/mwh e* (FM7 females) were collected from the mating described above. These females were collected as eggs from 24 hour layings and were divided into 19 batches for treatment. The eggs were collected and reared in 1/2 pint bottles on medium containing 100 µg/ml of chloramphenicol. Three batches served as controls, one continuously incubated at 22°, one continuously incubated at 29° and one given a 72 hour 22°/48 hour 29° treatment without irradiation. A further 8 batches were given 72 hour 22°/48 hour 29° treatments and were irradiated with 1500 rads at different times in development to induce somatic recombination. The remaining 8 batches were continuously incubated at 22° and were irradiated at the same times with the same dose as the 29° treated groups.

Eclosing 726 and FM7 females from each batch were collected, counted and the 726 females scored for DFP's under the dissecting microscope as described in the materials and methods. The eclosed 726 females

with DFP's and samples of the 726 females incubated at 22° continuously, both irradiated and control, were then prepared for scoring under the compound microscope as described. The uneclosed pharate adults were counted and a sample of 150 from each batch was dissected from the pupal cases and scored under the dissecting microscope. The 726 female survival and DFP frequencies were calculated using these samples, as described in the materials and methods.

The DFP and Survival Frequencies

The DFP and survival frequencies for the 726 females from each batch are given in Table 19. No 726 females survived in the 29° control, and the 22° control shows 100% relative survival and no DFP's, as expected. The 29° treated control shows a reasonable DFP frequency (24.5%) and a lowered survival (63.5%). The DFP frequency is higher than this (39.9%) and the survival lower (47.3%) in the batch irradiated at 24 hours. This is likely due to the interaction between the effects of the irradiation and the 29° treatments noted in the previous section. This is supported by the observation that the batches incubated at 22° continuously and irradiated at 24 and 48 hours had a small number of DFP's (1.0% and 0.7% respectively). None of the other 22° continuous irradiated batches had any DFP's and the 726 survival relative to the FM7 controls was 100% or greater in all of these batches.

Scoring of Clones

Somatic recombination induced by a radiation treatment (Becker 1957) or occurring spontaneously in the X chromosomes of imaginal disc cells of a 726 female will often produce twin spots or single clones, depending on the location of the recombination event along the X chromosome, as described in the materials and methods (Figure 1). Samples of the meso-

Table 19 The Frequencies of Survival, Eclosion and DFPs among *726* Females Reaching the Pharate Adult Stage

	Time of irradiation (hr)	FM7 Females		726 Females		Survival of 726 Females (%)[1]	DFP Mesothoracic legs	
		Number	Frequency eclosed (%)	Number	Frequency eclosed (%)		Number	(%)
Irradiation	24	2365	72.3	1119	33.6	47.3	892	39.9
and	48	3083	82.0	1896	70.0	61.5	986	26.0
48 hour	96	2413	91.5	1495	77.6	62.0	668	22.3
29°	120	3037	92.0	1692	76.5	55.7	788	23.3
treatment	144	2699	89.5	1343	78.0	51.6	827	30.8
	168	3040	93.3	1877	73.8	61.7	962	25.6
	192	2482	93.0	1733	72.0	69.8	984	28.4
	216	2100	91.4	1502	76.0	71.5	824	27.4
	TOTAL	21117	93.5	12657	70.9	59.9	6985	27.6
No irradiation and:								
48 hour 29° treatment	-	2240	93.5	1365	78.4	63.5	669	24.5
29° control	-	3173	93.5	0	-	0	-	-
22° control	-	1607	91.0	1667	94.1	100	0	0

[1] Relative to FM7 females

thoracic legs from the 726 females continuously incubated at 22° (referred to as 22° control legs) of the morphologically normal legs from the 29° treated 726 females with a single DFP (referred to as 29° treated control legs) and of the DFP's were scored under the compound microscope for the presence of such clones. The 29° treated legs served as an internal control for the effects of the 29° treatment on imaginal disc cells. The clone frequency was recorded for each leg type in each batch as the mean number of clones per leg.

Each clone in the DFP's was first scored for location. Those clones marking structures in the duplicate portion of the DFP only were classified as *D* clones. Those marking only structures in the original portion of the DFP were classified as *O* clones. Those marking structures in both portions were classified as *OD* clones. Each clone was then scored for the number of marked elements (trichomes and bristles) present.

Subtraction of Spontaneous Clones

The following analysis depends on measuring the difference between the clones induced by each radiation treatment, however, in each batch some clones will occur spontaneously. Earlier clonal analyses (Garcia-Bellido and Merriam 1971) have shown that most spontaneous clones occur late in development and are small. The inclusion of such clones will tend to decrease the mean clone size in those batches irradiated early, while those batches irradiated later will be less strongly affected. In an attempt to eliminate this source of error, the distribution of spontaneous clones expected in each treatment batch was subtracted from the data set.

Samples of unirradiated 22° control legs, 29° treated legs and DFP's were scored for the presence of spontaneous clones. These clones occurred

with low frequencies (Table 20) and were variable in size, with the majority being small (eg., of 19 clones in unirradiated 22° control legs 14 had 1-5 *mwh* trichomes, 3 had 5-10, 1 had 54 and 1 had 150). Accordingly, the spontaneous clones for each leg type were divided into size classes with the class intervals chosen so as to reflect the number of cell divisions following induction of the clones. A frequency distribution for each size class within each leg type was then plotted. Finally, the expected number of spontaneous clones in each size class for each sample of irradiated legs was calculated, and these numbers were subtracted from the corresponding numbers of clones observed in each size class after irradiation. The frequency distributions for the spontaneous clones, subdivided according to size are given in Appendix D.

The Frequencies of Induced Clones

The frequencies of clones in each irradiation treatment batch after subtraction of the spontaneous distribution are given in Table 21 for the 22° control and 29° treated legs and in Table 22 for DFP's. To facilitate comparison between the clone frequencies, the clone frequency at each irradiation time was plotted for the legs in each treatment class. The results of earlier clonal analyses (Bryant and Schneiderman 1969, Garcia-Bellido and Merriam 1971) indicate that in normal imaginal discs the clone frequency increases exponentially with the time of treatment. Assuming this also to be the case here, the clone frequencies were plotted on semi-logarithmic paper as shown in Figure 21 for the controls and in Figure 22 for DFP's. The lines in these figures were fitted to the data points as described below.

The 22° control leg clone frequency data show a good fit to a linear increase with time of irradiation when plotted on semi-logarithmic paper,

Table 20 The Frequency of Spontaneous Clones in 22° Control Legs, 29° Treated Control Legs and in DFPs

Clone Type	Number of Legs	Number of Clones	Frequency of Clones
22° Control	250	19	.08
29° Treated control	185	16	.09
O Clones	231	20	.09
D Clones	231	13	.06
OD Clones	231	0	0

Table 21 The Frequency of Induced Clones in 22° Control and 29° Treated Control Legs

29° Treated Control Legs

Larval age at irradiation (hr)	Number of legs	Number of clones	Frequency of clones
24	92	16	.17
48	82	15	.18
96	58	22	.38
120	108	41	.38
144	57	26	.46
168	36	36	1.00
192	19	52	2.74
216	9	113	12.56

22° Control Legs

Larval age at irradiation (hr)	Number of legs	Number of clones	Frequency of clones
24	254	32	.13
48	71	8	.11
96	75	56	.75
120	31	78	2.52
144	24	80	3.33
168	16	276	17.25
192	6	200	33.33
216	6	405	67.50

Table 22 The Frequency of Induced Clones in DFP Legs Marking Original Structures Only (O), Both Original and Duplicate Structures (OD) and Duplicate Structures Only (D)

Clone Type	Larval age at Irradiation (hr)	Number of Legs	Number of Clones	Frequency of Clones
O	24	64	9	.14
	48	60	16	.26
	96	61	23	.37
	120	136	41	.30
	144	95	39	.42
	168	58	49	.84
	192	37	60	1.62
	216	11	111	10.09
OD	24	142	6	.04
	48	153	5	.03
	96	177	2	.01
	120	154	1	.01
	144	159	1	.01
	168	94	0	0
D	24	142	10	.07
	48	153	13	.08
	96	177	15	.08
	120	154	20	.13
	144	159	21	.13
	168	94	23	.24
	192	38	26	.68
	216	12	87	7.25

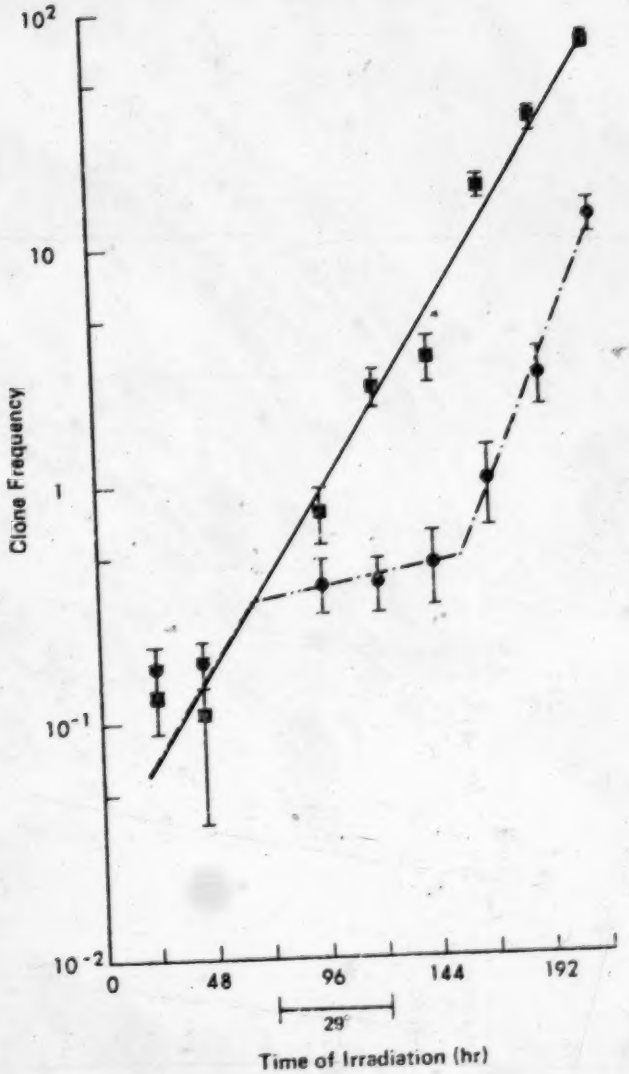


Figure 21 The frequency of induced clones in 22° control legs ■——■ and 29° treated control legs ●- — — -●
expressed as the mean number of clones per leg.
95% confidence intervals are shown about each point.

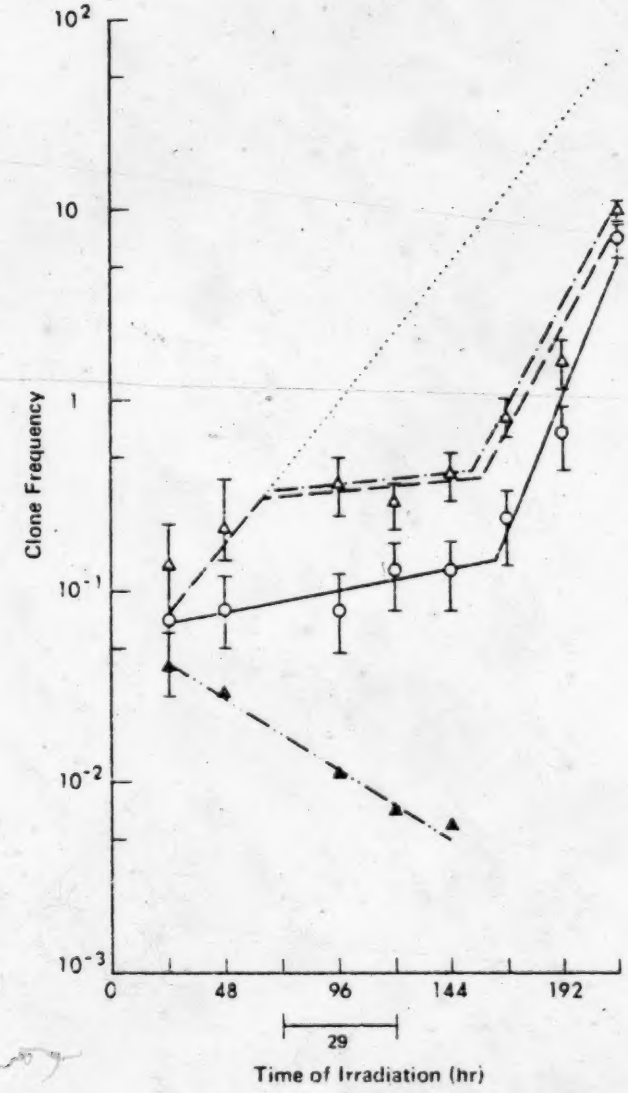


Figure 22 The frequencies of clones marking only original portions (O) Δ— — —Δ , duplicate portions (D) O——O , and both portions (OD) ▲——·——▲ of DFPs expressed as the mean number of clones per leg. 95% confidence intervals are shown for each point. The curves for the 22° control leg ..... and 29° treated control leg -·-·- clone frequencies taken from Figure 20 are included for comparison.

as expected if the clone frequency is increasing exponentially. Linear regression was therefore used to fit a line to these data points. First the clone frequencies were transformed to logarithms, and then a linear regression line was fitted to the data points by the least squares method (Snedecor and Cochran 1967). The regression coefficient for this line is given in Table 23. As a test of the assumption that the transformed clone frequencies show a linear relationship with the time of irradiation, the proportion of the variation about the mean explained by the regression, r^2, was calculated. For this line, r^2=97.7% indicating that the residual variance is very small, and thus that the assumption is validated.

The clone frequencies in the 29° treated control legs do not show a linear increase with the time of irradiation when plotted on semi-logarithmic paper. As might be expected the frequencies are not significantly different from the 22° control clone frequencies at 24 and 48 hours (i.e., before the 29° treatment) but they begin to deviate from control frequencies by 96 hours. The data suggest that the 29° treated control leg clone frequencies do not increase significantly between 96 and 144 hours. This is the period during and immediately following the 29° treatment. After 144 hours, however, the log of clone frequency appears to increase linearly with larval age at irradiation, at a rate similar to that in the 22° controls. Inspection of the data therefore suggests that the clone frequencies are affected by the 29° treatment, but that the effect does not last more than 24 hours beyond the return to 22°.

In an attempt to confirm these conclusions, separate regression lines were fitted to the 96-144 and 168-216 hour points (regression coefficients

Table 23 Regression Coefficients for Clone Frequency and Clone Size in 22° Control Legs, 29° Treated Control Legs, and DFPs

Type of Line	Type of Clone	Hours	Regression Coefficient
Clone frequency	22° Control	24-216	0.0355
"	29° Treated	96-144	0.0398
"	29° Treated	168-216	0.0527
"	O	96-144	0.0264
"	O	168-216	0.0518
"	D	96-144	0.0101
"	D	168-216	0.0710
"	OD	24-144	-0.0246
Clone size	22° Control	24-216	-0.0342
"	29° Treated	96-144	-0.0237
"	29° Treated	168-216	-0.0433
"	O	96-144	-0.0230
"	O	168-216	-0.0518
"	D	96-144	-0.0113
"	D	168-216	-0.0623

in Table 23). Two tests were done on these lines. First, the lines were tested for similarity following the procedure outlined in Snedecor and Cochran (1967, ch. 14, pp. 432-436). No differences were found in the residual variances ($P>.25$), however, the regression coefficients were significantly different ($P<.025$). Secondly, the 160-216 hour line was tested against the 22° control leg clone frequency regression line. Neither the visidual variance ($P>.25$) nor the regression coefficients ($P>.10$) were significantly different. These results support the conclusion that the clone frequency in 29° treated control legs increases at the normal rate from 168 to 216 hours. Thus the imaginal disc cells appear to recover their original rate of cell division between 24 and 48 hours after the end of the 29° treatment.

Clone frequency has been used as an indicator of the relative numbers of cells present in the imaginal disc (Garcia-Bellido and Merriam 1971). The lack of increase in clone frequency between 96 and 144 hours can thus be taken to indicate that the total number of cells present in the 29° treated leg disc does not increase significantly during this period. The possible reasons for this are further investigated below.

The frequencies of the three types of clones in DFP's, O, D and OD, were also plotted on semi-logarithmic paper (Figure 22). The regression lines for the two types of morphologically normal legs are indicated on this figure for comparison. One observation that can readily be made is that the O clone frequencies are very similar to the 29° treated leg clone frequencies. Consideration of the 95% confidence intervals suggests that there is no significant difference between the frequencies of these two types of clones at any irradiation time. Regression lines were fitted to the O clone frequencies between 96 and

144 hours and between 168 and 216 hours (Table 23). As might be expected the regression coefficients of these lines were not significantly different from the 29° treated leg clone lines ($P>.50$, $P>.90$ respectively).

The D clone frequency is substantially lower than the O clone frequency at every irradiation time after 24 hours. In fact, the D clone frequency shows no significant increase between 24 and 144 hours. Regression lines were fitted to the 24-144 hour and the 168-216 hour sets of points (Table 23). These lines were compared with the 96-144 hour and 168-216 hour O clone frequency lines respectively. The lines were not significantly different in slope ($P>.50$, $P>.50$) but were significantly different in elevation ($P<.01$, $P<.05$)

In contrast to all other clone frequencies, the OD clone frequency declines with increasing age at irradiation between 24 and 144 hours. No OD clones were observed in batches irradiated later than 144 hours. A regression line was fitted to the OD clone frequencies (Table 23). No confidence intervals are shown for the OD points as there were only 4 OD clones in the 96-144 hour treatment batches.

These observations can be related to the original hypothesis of epimorphosis in the classification of the portions of the DFP's. According to this hypothesis, the original portion is expected to resemble a 29° treated leg, while the duplicate is expected to have fewer cells, and hence a lower clone frequency, both of which occur. In addition, observation on the O, D and OD frequencies can be related to the question of the timing of duplicate initiation and growth. First, the lack of OD clones in the batches irradiated later than 144 hours implies that the two portions of the DFP are separate entities after 144 hours. Caution must be used in interpreting these data as the

natural decrease in size of clones induced at later irradiation times will make the scoring of OD clones difficult. However, it seems reasonable to conclude that no significant recruiting of original cells into the duplicate occurs after 144 hours.

The inflection point in the duplicate clone frequency curve after 144 hours indicates that the duplicates begin to increase in cell number at about the same time as the unduplicated legs and the original portions resume normal growth. The previous observation on the OD clones supports the conclusion that this increase is not due to recruitment of cells from the original portion, but rather is the result of cell divisions within the duplicate. The insignificance of the difference between the regression coefficients for the O and D clone frequency lines between 168 and 216 hours implies that duplicates grow at about the normal rate.

Taken together these results suggest that the duplicate is initiated by a small number of cells at or before 144 hours which begin to grow shortly thereafter. If this is so, then the D clone frequency at 144 hours should reflect the number of cells present in the newly initiated duplicate. At 144 hours the D clone frequency is 13%. This is comparable to the O clone frequency and the 22° leg clone frequency at 24 hours (14% and 13% respectively), suggesting that the number of cells in the duplicate at initiation is comparable to the number in normal legs early in development.

Clone Size

Only those clones marking trichomes (*mwh sn*^{3} or *mwh*) were used in the calculation of clone size. This is because there are only about 450 bristles on the mesothoracic leg surface so that a clone marking only

bristles has a minimum size of 1/450 of the leg (Bryant and Schneiderman 1969). This, coupled with the uneven distribution of bristles on the leg surface will strongly affect the accuracy of any calculations of the mean size of bristle clones. The much greater number of trichomes, and their more even distribution on the leg surface greatly reduces this problem, although it does not completely eliminate it. There are areas of the leg that do not contain either trichomes or bristles and hence could not be scored for clones (Figure 3).

The clones were originally scored for the number of trichomes and bristles marked. This number was converted into a fraction, or relative measure of the clone size so that estimates of the number of cells present at the time of irradiation could be made. To make this conversion, the number of marked elements in each clone was divided by the total number of trichomes and bristles in the leg. Accordingly, samples of the 22° legs, the 29° treated legs and the DFP's were scored for the numbers of trichomes and bristles present. The samples contained only legs or DFP's with a clone. The numbers of legs scored and the mean numbers of elements present are shown in Table 24. As was noted previously, the duplicate portions of the DFP's were on the average much smaller and more variable in size than the originals, the 22° control or the 29° treated legs.

To eliminate error caused by this variation, the number of trichomes and bristles marked by each D or OD clone was divided by the total number of trichomes and bristles in the duplicate, or DFP respectively in which the clone occurred. This could be done as each duplicate with a clone was included in the sample scored for trichomes and bristles. Averaging the relative sizes for all of the clones in

Table 24 Means and Standard Deviations(s) of the total number of Trichomes and Bristles from Samples of Control Legs and DFPs

Leg Type	Mean number of trichomes	s	Number of cases	Mean number of bristles	s	Number of cases	Mean number of trichomes and bristles	s
Original portion of DFP	12716.8	230.8	35	390.1	28.3	73	13106.9	243.8
Duplicate portion of DFP	4795.8	2135.0	176	230.4	97.6	176	5026.2	2177.0
29° Treated Control	19020.0	170.8	37	432.4	12.8	76	19452.4	176.5
22° Control	22371.5	61.2	40	486.3	7.2	71	22857.8	64.5

a radiation treatment batch gave the mean relative clone size for that batch.

The mean relative clone size for 0 clones and for the clones in 22° control legs and 29° treated control legs was calculated by dividing each clone by the mean number of trichomes and bristles in the sample of legs scored. Approximating each of the legs by the appropriate sample mean will not induce a great deal of error, given the smaller standard deviation in the sample, and it eliminates the necessity of scoring a large number of these large legs for trichomes and bristles.

The mean relative sizes of the clones in each irradiation batch are given in Table 25. To facilitate comparison the mean clone sizes were plotted on semi-logarithmic plots, the 22° control and the 29° treated leg clone sizes are shown in Figure 23, and those of clones in DFP's in Figure 24.

A single regression line was fitted to the 22° control clone size points. As with the clone frequency, the points showed a reasonably good fit to this line (r^2=96.7%). Inflection of the 29° treated leg clone size data points again reveals clear evidence that the curve is broken into three distinct segments, 24-48, 96-144 and 168-216 hours. No change in clone size occurs between 24-48 hours while the 96-144 and 168-216 hour periods show different rates of decrease. Regression lines were fitted to data in the latter two periods. Tests indicate that the slopes of these two lines are different ($P<.05$) and that there is no difference between the slope of the 168-216 hour line and that of the 22° leg clone sizes ($P>.20$). The regression coefficients are given in Table 23.

One observation that can be made from these data is that the mean

Table 25 Mean Relative Clone Sizes in 22° Control Legs, 29° Treated Control Legs and DFPs, Expressed as a Percentage of Total Leg Size.

Larval age at Irradiation	22° Control[1] Leg Clones	29° Treated[1] Control Leg Clones	O[1] Clones	D[2] Clones	OD[3] Clones
24	3.41	2.22	3.73	13.92	.09
48	1.40	2.22	3.00	15.84	.05
96	.48	1.06	1.57	12.88	.03
120	.13	.52	.79	10.56	.03
144	.07	.34	.52	7.48	.04
168	.02	.16	.30	2.39	0
192	.01	.04	.11	.60	0
216	.006	.02	.04	.12	0

1. Calculated as

$$1/n \sum_{i=1}^{n} \left[\frac{\text{The number of } mwh \text{ trichomes + } sn \text{ bristles in each clone}}{\text{The mean number of trichomes + the mean number of bristles in the leg}} \right] \times 100$$

2. Calculated as

$$1/n \sum_{i=1}^{n} \left[\frac{mwh \text{ trichomes + } sn \text{ bristles in each O clone}}{\text{Total trichomes + bristles in that duplicate}} \right] \times 100$$

3. Calculated as

$$1/n \sum_{i=1}^{n} \left[\frac{mwh \text{ trichomes + } sn \text{ bristles in each OD clone}}{\text{Total trichomes + bristles in that DFP}} \right] \times 100$$

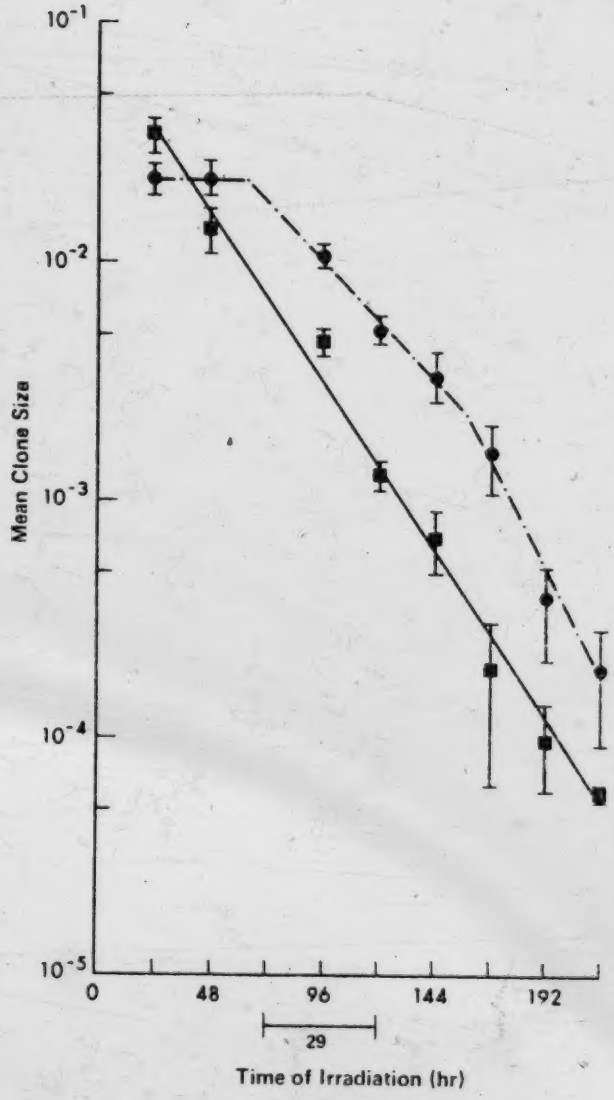


Figure 23 The mean relative sizes of clones in 22° control ■——■ and 29° treated control ●- - - -● legs. Calculated as the number of elements in a clone divided by the number in the corresponding leg. See Table 25 for the exact formulae used; 95% confidence intervals are shown for each point.

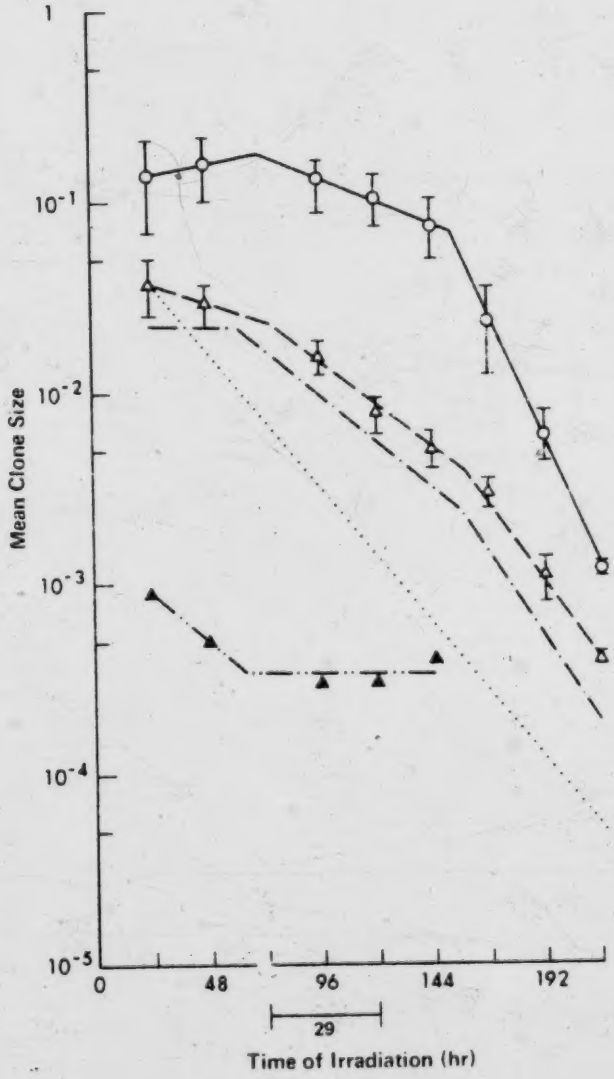


Figure 24 The mean relative sizes of O clones △ — — △ D clones ○———○ and OD clones ▲·—··—▲ . The curves for the 22 control ..... and 29 treated control —·—·· leg clones are given for comparison. 95% confidence intervals are given for each point.

relative size of 29° treated control leg clones decreases significantly between 96 and 144 hours. Comparison with the clone frequency curve for the same period suggests that the rate at which the clone frequency increases is much less than the rate at which the clone size decreases. In normal imaginal discs the decrease in relative clone size during development is matched by the increase in clone frequency (Garcia-Bellido and Merriam 1971). The observations on the 29° treated control leg clone sizes suggest that the portion of adult structures formed by the descendants of a single cell declines during the 29° treatment, as it does during normal development, where as the clone frequency data indicates the overall number of cells is not increasing. One reasonable hypothesis as to how this occurs would be that the cell division rates of the imaginal disc cells at 29° are nearly matched by the rate of induction of cell death.

The mean relative O clone sizes are strikingly similar to the 29° treated leg clone sizes, as might be expected given the similarity of the clone frequencies. Two linear regression lines were fitted to the O clone size points, one to the 96-144 and the other to the 168-216 hour points. F tests demonstrate that neither the slope ($P>.40$, $P>.50$) nor the elevation ($P>.60$, $P>.50$) of these lines is different from the corresponding lines for the 29° treated control leg clones.

The mean relative D clone size is greater than the O clone size at every irradiation time. As with the clone frequencies the D clone sizes do not significantly change from 74 to 144 hours, and decrease rapidly thereafter. Accordingly, two linear regression lines were fitted to these points, one to the 24-144 hour points, the other to the 168-216 hour points. F tests indicate that the slopes of these lines

are different ($P<.01$) and that the slopes of these lines are not different from those of the corresponding lines for the O clones ($P>.10$, $P>.50$ respectively), although the elevations of the lines are different ($P<.01$, $P<.05$). The line fitted to the OD clone size points is not a regression line, but was simply drawn to indicate the trend of the OD clones. These clones were small and, as indicated earlier, infrequent after 48 hours.

The observations on the 29° treated leg, the O and the D clone sizes provide further support for the hypothesis that duplication is occurring by epimorphosis. According to this hypothesis the 29° treated leg and O clones should be similar in size while the relative size of D clones should be larger than either. This is what is observed.

Estimates of the number of cells present in each leg or DFP portion were made using the clone size data. First, the number of cells present at each irradiation time was estimated at 1/2 of the inverse of the mean relative clone size (Becker 1957, Bryant and Schneiderman 1969, Garcia-Bellido and Merriam 1971). In the DFP's, only O and D clones were used to estimate the number of cells in original and duplicate portions respectively. The OD clones were not used as only a part of each OD clone marks each portion of the DFP.

The OD clones and the D clones were used in a second calculation to estimate the number of cells which become committed to form the duplicate was marked by the clone. The inverse of the smallest relative clone size in each treatment was therefore calculated. If the assumption is correct, this will estimate the number of initial cells. This is analogous to the smallest patch size analysis done on gynandromorphs (Stern 1963, see also Wieschaus and Gehring 1976b). There have been

criticisms of this technique (ibid). Since the estimate is based on one extreme of the population of clones, fluctuations in the local growth rates with the disc will tend to make this an overestimate. However, the estimation was done to allow comparison between the duplicate portions of DFP's and estimates of initial cell number in normal legs at blastoderm done using this method (see Madhavan and Schneiderman 1977 for review).

The resulting estimates of cell number are given in Table 26. The estimates for the 22° legs and the 29° treated legs are illustrated in Figure 25. The estimates of cell number in the original and duplicate portions of the DFP are illustrated in Figure 25. In addition, the smallest clone estimates for the initial cell number are shown in Figure 26 for the 24-144 hour irradiation batches.

Several observations can be drawn from these cell number estimates. First, the number estimated to be in the 22° control legs at 24 hours is similar to other estimates of the number of cells in normal legs at this stage in development (Madhavan and Schneiderman 1977 for review). Secondly the number in 29° treated control legs is somewhat higher than this at 24 hours. This may be due to the cell death decreasing the size of the clones induced at 24 hours. If, as indicated previously (Clark 1976), cell death is induced by *ts726* in contiguous patches, small clones will most often be either completely removed or unaffected by the death while large clones will more often be partially removed.

Thirdly, the number of cells estimated to be in the duplicate at 144 hours (6.7) is considerably smaller than in the original at that time (96.1), supporting the hypothesis of epimorphosis. Fourthly, the estimates of cell number in the duplicate at initiation are very similar

Table 26 Estimates of the Number of Cells in Control Legs and DFP Portions at each Irradiation Time[1]

Time of irradiation (hr)	Estimates of Cell Number from Average Clone Size				Estimate of cell number from the size of the smallest type D clone	
	Original portions of DFPs	Duplicate portions of DFPs	29^{o} treated control legs	22^{o} constant control legs	Smallest clone size	Estimated cell number
24	13.4	(2.4)	22.5	14.7	.05	20.0
48	16.7	(3.2)	22.5	35.7	.04	23.3
96	31.8	(3.9)	47.0	105.3	.05	19.6
120	63.3	(4.7)	95.4	384.6	.04	26.0
144	96.1	6.7	145.0	714.3	.02	(62.1)
168	166.5	20.9	319.4	2645.5	.0046	(217.4)
192	476.2	83.3	1187.4	4948.2	.0012	(813.0)
216	1306.7	416.7	2360.7	8386.0	.00014	(7246.4)

1. Values in parentheses are those time points at which the particular estimate is inappropriate as a measure of cell number (see text)

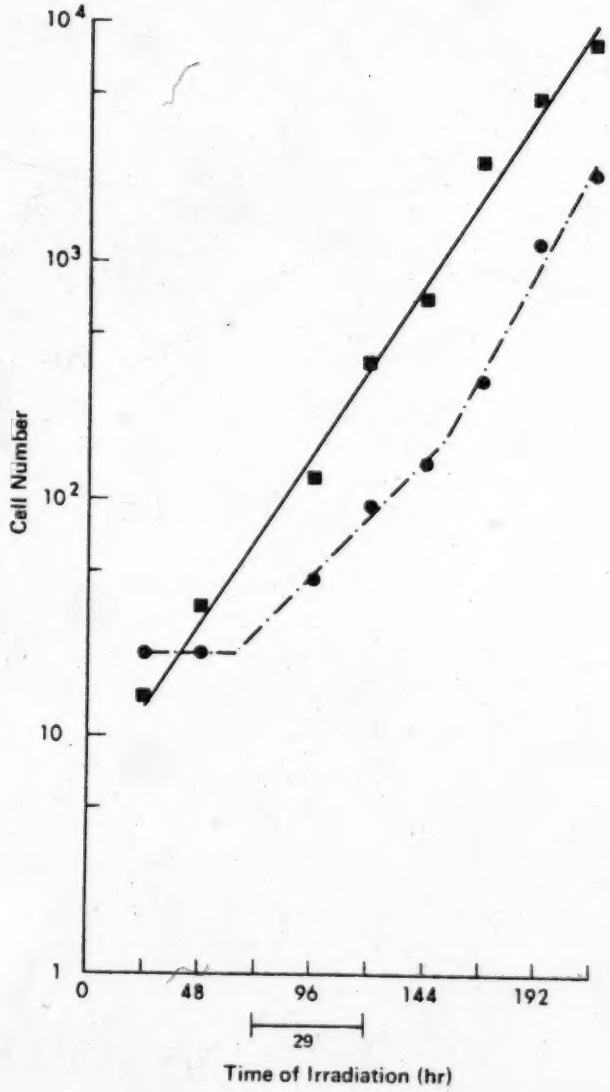


Figure 25 The number of cells in the 22° control ■——■ and 29° treated control legs ●— — —● , estimated at each irradiation time as half the inverse of the mean clone size.

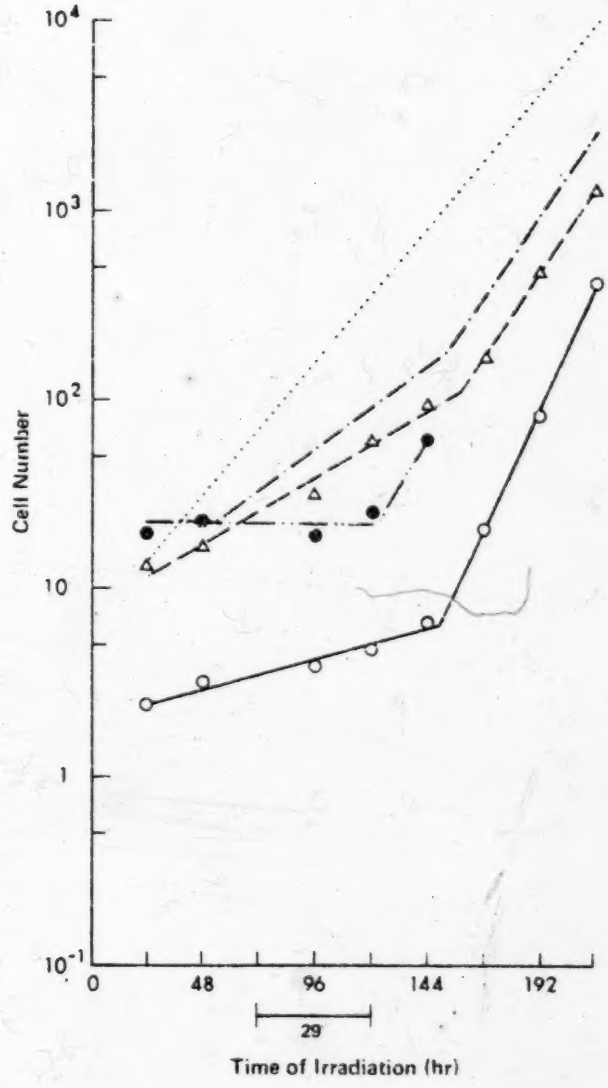


Figure 26 The number of cells in the original △— — —△ and duplicate ○————○ portions of DFPs estimated as half the inverse of the mean clone size. Also, the number of cells estimated to initiate the duplicate, estimated as the inverse of the smallest relative clone size in each irradiation batch ●···—···● is shown for the 24-120 hour batches. The cell number curves for the 22° control ····· and the 29° treated control —·—·— legs from Figure 24 are shown for comparison.

to the estimates of cell number in normal legs at blastoderm. The smallest clone estimate remains constant at about 21 cells until 120 hours. This is close to the estimates of 20 cells for normal legs derived from analyses gynandromorphs (Madhavan and Schneiderman 1977 for review). The estimate of 6.7 cells from the mean clone size at 144 hours is similar to estimates made on normal legs (7-10 cells) using the same calculation on normal leg clones induced in embryos (ibid). In addition, the rise in the smallest clone size estimate at 120 hours indicates that the smallest clone induced at this time was not induced prior to the initiation of the duplicate. Since one cell division is required to segregate the initial marked cell of a clone, this means that in at least some duplicates cells are committed to form the duplicate within one cell division after the return to 22°. The rise in the cell number estimate calculated from the mean clone size at 168 hours indicates that growth begins in most if not all of the duplicates within 48 hours after the return to 22°.

Estimation of the Amount of Cell Death in 29° Treated Legs

The clone frequency and clone size data presented above for the 22° control legs and the 29° treated legs can be used to make an estimate of the amount of cell death induced by *ts726* in the 29° treated legs. The difference in clone frequency between these two leg types at 144 hours is presumably due to the difference in the number of cells present. This difference is most likely the combined result of cell death and a reduced rate of cell division of the surviving cells at 29°. These components can be separated by considering the corresponding data for clone size. In normally developing legs the rate of increase in clone frequency is matched by the rate of decrease in clone size

(Garcia-Bellido and Merriam 1971). The cell cycle time can thus be estimated from the slopes of either of these curves, as demonstrated by the 22° control legs in the present study (19.6 hours from clone frequency and 20.0 hours from clone size).

The clone frequency and clone size curves from the 29° treated legs do not show this reciprocal relationship between 72 and 144 hours. The clone size decreases much faster than the clone frequency increases. The two calculations based on the slopes of these curves do not agree (30 vs. 230 hours) and hence cannot be taken as estimates of the cell cycle time. This situation is what might be expected if cell death and a reduced rate of cell division in surviving cells are both occurring at 29°. If the cell death were highly localized, as is indicated by histological evidence (Clark and Russell 1977), then cells in the surviving regions of the disc would continue to undergo somatic recombination and to produce clones. Thus the clone frequency could reflect the net effect of cell death and cell division on the size of the surviving cell population. If these two effects are nearly equal, the frequency of clones induced by X-rays would fail to increase over time. This is actually the case during the 29° treatment in the results described above.

The rate of change of clone size, on the other hand, would depend largely upon the rate of cell division at 29°. Clones induced early in a population of cells undergoing both cell death and cell division would be larger on the average than clones induced later. This is because a localized pattern of cell death would usually lead either to elimination of a clone completely or leave its size unaffected. This will in fact depend on the relative sizes of the cell death patch, the clone and the

imaginal disc. If the clones are very large, i.e., occupy a substantial portion of the disc, then an appreciable number of the clones may partially overlap the cell death patch. If cases occur in which part of the marked cells are killed and the remainder are not called on to regenerate, then the mean clone size will be lowered.

This large clone effect may be important for clones induced at 24 or 48 hours, but not for clones induced during the 29° treatment. These should be too small to be partially affected by the cell death patch with an appreciable frequency. Thus the difference between the rates of change of clone frequency and clone size between 72 and 144 hours will estimate the rate of cell death.

Since clone size decreases with time, i.e., has a negative slope, the sum of the slopes of the clone size curve and the clone frequency curve should represent the rate of decrease in cell number due to cell death. As shown in Table 23, these slopes are -0.027 and +0.0046 for the 29° treated control leg clones, giving a sum of -0.018. As the clone size and clone frequency lines were fitted to the logarithms of the data points, this represents the exponential rate of cell decrease. Using the formula $n_t=n_o \exp(rt)$, where n_t represents the final number of cells, n_o the original number of cells, r the rate of increase or decrease and t the time interval, the number of dead cells present in the 29° treated control leg can be estimated.

First, the number of cells which will survive the 29° treatment is calculated. The number of cells present in the 22° control leg at 72 hours can be estimated from the clone size curve as approximately 70 cells. The same number can be assumed to exist in the 29° treated leg at this time (i.e., before the 29° treatment). Substituting into the

equation gives an estimate of: 70 exp (-0.018x72 hours)=19 surviving cells. If these cells divided throughout the 29° treatment at the rate estimated from the clone size curve we expect: 19 exp (0.023x72 hours)= 98 live cells at 144 hours and 70-19=51 dead cells in the 29° treated control leg disc. This can be compared with the number of live cells expected had there been no cell death: 70 exp (0.023x72 hours)=362 cells at 144 hours.

The ratio of secondary lysosomes to cells reported by Clark from his study of 29° treated *ts726* eye-antennal discs was 18% (Clark and Russell 1977). This is less than the ratio of dead to live cells calculated above (51/98=52%). Three explanations for this difference are, first, that each lysosome might represent more than one dead cell. Secondly, the difference may be due to the fact that only one time point was sampled in the histological study, while the clonal analysis estimates are for the amount of cell death over the entire period between 72 and 144 hours. Thirdly, there may be an inherent difference in the amount of cell death which occurred in these second leg discs in this *ts726* stock following this 29° treatment and in the eye-antennal discs studied by Clark.

Investigation of the Variation in Duplicate Completeness

The previous analyses of the somatic cell clones in the duplicate portions of DFP's implicitly assumed a uniform population of duplicates at each irradiation time. However, it has been noted previously that there is considerable variation in the completeness of the duplicates after metamorphosis. The stage in development of the duplicates at which this variation arises is thus an important factor in the interpretation of the clone data. For example, it would be important to

know whether the variation in duplicate size found after metamorphosis reflects variation in the size of the cell populations initially committed to form the duplicate after the heat treatment. The clone data and the data on the incompleteness of duplicates was therefore examined for correlations that might indicate when this variation arises.

Three hypotheses were considered. First, the time of initiation of duplicate growth might vary, with each duplicate initiating growth at a random time after the 29° treatment. Those duplicates which initiated growth early would be more complete than those initiated later. Secondly, the time of termination of duplicate growth may vary. Each duplicate may begin to grow at about the same time, but might stop growing at random times thereafter. Those which continue to grow the longest would be the most complete. Thirdly, the variation may be the result of differences in the number of cells that initiate the duplicates. If cell divisions occur at the same rate in all of the growing duplicates, those initiated with the largest number of cells would be the most complete.

The first two hypotheses were tested by examining two correlations. First between the number of trichomes and bristles in duplicates with clones and the time of irradiation, and second between the number of trichomes and bristles in the duplicates with clones and the number of trichomes and bristles marked by these clones. The number of trichomes and bristles in a duplicate was used as a quantitative measure of completeness of the duplicate. According to the first hypothesis, irradiation shortly after the end of the 29° treatment should induce D clones in only those duplicates which had begun to grow early, i.e., those which will be the most complete. A later irradiation, however, will

also induce D clones in incomplete duplicates. Thus the mean number of trichomes and bristles in duplicates with clones should be lower in later irradiation batches. Thus under this hypothesis a negative correlation between the time of irradiation and the trichomes and bristles in the duplicates with clones is predicted.

A second prediction from this hypothesis was tested by examining the second correlation. At any one time after the 29° treatment this hypothesis predicts that all of the duplicates will continue to grow for about the same amount of time. Thus all of the D clones induced in these duplicates will go through about the same number of cell divisions and will mark about the same number of trichomes and bristles, independent of the completeness of the duplicates. This corresponds to a prediction of no correlation between the number of trichomes and bristles marked and the total number of trichomes and bristles in the duplicates in each irradiation treatment.

The second hypothesis, that the variation arises through differences in growth termination, predicts different results from these correlations. First, radiation given shortly after the end of the 29° treatment should induce D clones in all duplicates with a similar frequency, as they should all contain about the same number of cells. However, a later irradiation will only induce D clones in those duplicates that are still growing. These will be the more complete duplicates. Thus a positive correlation between the number of trichomes and bristles in the duplicates and the time of irradiation is predicted.

Secondly, according to this hypothesis, at any one time after the 29° treatment the amount of time each duplicate has left to grow will vary. Thus clones induced in those duplicates with a longer time to

grow will go through more cell divisions and mark more trichomes and bristles. Thus the more complete duplicates will have larger clones. This leads to a prediction of a positive correlation between the number of trichomes and bristles in the duplicate and the number in the clones, within an irradiation batch.

The third hypothesis was tested by examining the correlation between the size of the duplicates and the relative clone sizes within irradiation treatment batches. Each clone is initiated by 1 cell and, assuming marked cells divide at the same rate as unmarked cells, the relative clone size or fraction of the duplicate marked will not change throughout development. This fraction can thus seem as an indicator of the number of cells present at the time of irradiation. According to this hypothesis, those duplicates with the most cells at initiation will have the most cells at irradiation and will thus have the smallest relative clone size. These will also be the most complete duplicates. This gives a prediction of a negative correlation between the relative clone size and the duplicate size within a radiation treatment batch.

The results of the correlation analyses are given in Table 27. Both the correlation of the duplicate size with the irradiation time and with the number of trichomes and bristles in a clone are positive and significant. This is in agreement with the predictions of the second hypothesis. In addition the correlation between the duplicate size and the relative clone size is neither consistantly negative nor significant, which argues against the third hypothesis.

A variety of other models could probably be invented to account for the observed data, particularly in view of the fact that the models tested above are not mutally exclusive. However, the hypothesis that

Table 27 Coefficients of correlation (r) between the number of duplicate leg elements (A) and the time of irradiation (B), the number of marked elements in a clone (C) and the relative clone size (D) among duplicated legs with clones.

	Pearson Correlation Coefficient	Prob.[1]
r_{AB} (from 120-216 hr irradiation treatments)	+ 0.65	0.001
r_{AC} (from the 120 hr irradiation)	+ 0.68	0.03
r_{AC} (from the 144 hr irradiation)	+ 0.72	0.01
r_{AD} (from the 120 hr irradiation)	+ 0.17	0.57
r_{AD} (from the 144 hr irradiation)	- 0.24	0.31

[1] Level of significance of the deviation of r from zero.

the duplicates are all initiated at about the same time after a 29° treatment with approximately the same number of cells and that the observed variation in final duplicate completeness arises through differences in the time of termination of duplicate growth is favored by the results. This model also seems most plausible in view of what we know of the biology of the *ts726* system.

Clonal Restrictions in the Formation of DFP's

The disappearance of OD clones at 144 hours in the previous results indicates that the two portions of the DFP become separate at about this time. However, the OD clones are small and scarce after 48 hours so this separation may simply reflect the technical limitations of the analysis. Thus the question as to whether the original and duplicate are separate has not yet been fully answered. Secondly there is the question of the compartmentalization of the duplicate. It has been shown that after a certain time clones will not grow across certain boundaries in normally developing imaginal discs (Garcia-Bellido et al. 1973, Garcia-Bellido 1975). In the mesothoracic leg such a boundary has been shown to divide the anterior from the posterior (Steiner 1976). A natural question to ask is whether this boundary also exists in duplicates.

These two questions were investigated by a clonal analysis using the *Minute* technique (Garcia-Bellido et al. 1973). In this technique marked clones which are also *Minute*$^+$ are induced in a heterozygous *Minute* background. Since heterozygous *Minute* cells grow more slowly than normal, the clones grow very large (Morata and Ripoll 1975). The large size of the clones increases the precision with which compartment boundaries can be defined. In the present analysis the large clones were used to answer three questions. First, at what time does

the duplicate becomes separate from the original? Secondly, is there a compartment boundary formed in the duplicate? Thirdly, if a boundary forms in the duplicate, where and when does it form?

A number of *y w f ts726/FM7a;mwh/mwh* virgin females were mated to *y v f ts726/Y;Dp(1:3)Sc^{J4} y^{+} M(3)i^{55}/TM6* males to generate *y w f ts726/ y v f ts726;y^{+} M/mwh* females (called 726 *Minute* females) and *y w f ts726/ Y;y^{+} M/mwh* males (called 726 *Minute* males). Eggs from this mating were collected over 6 or 12 hour intervals and were divided into 11 batches for treatment. Two served as controls, one continuously incubated at 22° and the other at 29°. One batch was continuously incubated at 22° and irradiated at 48 hours with 1500 rads. The remaining groups were given a 102 hour 22°/48 hour 29° treatment and were irradiated at one of several times in development after the end of the 29° treatment. The 726 *Minute* females and males were given an increased length of time at 22° prior to the 29° treatment (102 vs. 72 hours for the 726 females) to compensate for their slower rate of development (Stern 1936, Steiner 1976).

The DFP's and a sample of the morphologically normal legs from the 22° irradiated 726 *Minute* females and males were prepared for scoring under the compound microscope as described in the materials and methods. They were then screened for the presence of clones. Somatic crossing over in the proximal portion of the right arm of the third chromosome in imaginal disc cells of these females and males will often produce a clone that is recognisable in the adult as a single spot containing trichomes marked with multiple wing hairs and yellow bristles (*mwh y*) as described in the materials and methods (Figure 2). The cells of such a clone will also be wild type for *Minute* and will grow at a faster

rate than the background cells, producing a large clone (Garcia-Bellido et al. 1973).

In addition to these large clones a sample of the twinspot clones from the previous experiment were scored in the determination of the compartment boundary in the duplicates. To facilitate comparison between the two experiments the times of irradiation were translated from hours after the midpoint of the egg collection into hours after the end of the 29° treatment.

The frequency of DFP's induced in, and the relative survival of heterozygous *Minute* females and males are given in Table 28. The *Minutes* show a lower DFP frequency than non-*Minutes*. This effect appears to be related to the *Minute* phenotype in some way, as the non-*Minute* sibs of these flies show a DFP frequency in the usual range (23.0% in the batch irradiated at 0 hours) after the 29° treatment.

Determination of the Duplicate Portion of the DFP

A sample of the DFP's from these 726 *Minute* females and males was screened for the presence of *mwh y Minute*$^{+}$ clones. The clones were first classified as being O, D or OD clones. The numbers of each of these types of clone in each irradiation batch are given in Table 29. Clones were scored as being OD only if a contiguous patch of marked tissue could be found which extended from the duplicate into the original. DFP's which had two separate patches of marked tissue, one in the original and a separate patch in the duplicate, were scored as having two separate clones.

The frequency of clones is higher in these DFP's than in the non-*Minute* DFP's examined previously. Comparison of the expected frequency of recombination as given in standard induced mitotic recombination

Table 28 The Relative Survival of and DFP Frequencies in *726 Minute* Males and Females

Developmental Stage at Irradiation (hr after 29° treatment)	Number of FM7 *Minute* Males	*726 Minute* Males				Number of FM7 *Minute* Females	*726 Minute* Females			
		Number of Adults	Relative Survival (%)	Number of DFPs	Percent DFPs		Number of Adults	Relative Survival (%)	Number of DFPs	Percent DFPs
0	1041	585	56.2	69	5.9	461	205	44.4	24	5.9
12	2106	1103	52.4	134	6.3	675	371	55.0	46	6.2
24	1502	798	53.1	72	4.5	543	277	51.0	34	6.1
36	1263	821	65.0	69	4.2	512	251	49.0	29	5.8
48	1185	694	58.6	55	4.0	921	402	43.6	49	6.1
60	1204	713	59.2	82	5.8	795	461	58.0	42	4.6
72	1411	762	54.0	61	4.0	528	256	48.5	31	6.1
	9712	5476	56.4	542	4.9	4435	2223	50.1	255	5.7

maps (Becker 1974) suggests that this difference cannot be accounted for by the differences in recombination frequency between the X and in the right arm of the third chromosome. A likely explanation for this difference is the well known increase in somatic recombination frequency associated with the *Minute* phenotype (Stern 1936, Morata and Ripoll 1973).

To answer the question as to when the duplicate becomes separate from the original, the number of OD clones present in each irradiation batch was examined (Table 29). Some OD clones appear in batches irradiated as late as 24-36 hours after the end of the 29° treatment, however, there is the possibility that these are not true OD clones but cases in which two or more clones have occurred which meet at the boundary of the O and D portions of the DFP. The *Minute*$^+$ clones in these DFP's were very large, so that an O and a D clone that both marked structures near the duplicate/original border might well be mistaken for an OD clone. This possibility was examined by comparing the number of observed OD clones to the number expected by the chance occurrence of two clones. This expected number was calculated as follows.

The probability of two or more clones occurring in one DFP which might resemble an OD clone can be calculated by multiplying the probability of one or more clones occurring in the original by the probability of one or more occurring in the duplicate. As these *Minute*$^+$ clones are very large, it is often impossible to tell whether a large patch of marked tissue is one or two clones. Thus, the probability of one or more clones is estimated as 1 minus the observed frequency of originals or duplicates with no clones. The calculation was done as follows. In a given irradiation batch, let o represent the number

Table 29 The Frequencies of O, D, and OD Clones Observed in, and the Number of OD Clones Expected in *726 Minute* DFPs

Developmental Stage at Irradiation (hr after 29° treatment)	DFPs #	Clones						
		O		D		Putative		Expected[1]
		#	freq	#	freq	OD #	(O + D)	OD
0	81	35	.43	14	.17	11	(5)	6.0
12	150	78	.52	32	.21	10	(10)	16.4
24	86	52	.60	27	.31	4	(10)	16.0
36	58	39	.67	21	.36	1	(12)	14.0
48	22	29	1.32	16	.73	0		
60	25	48	1.98	23	.92	0		
72	19	49	2.58	24	1.26	0		

1. Calculated as described in the text (p. 124).

of original portions of DFP's in which no clones were observed, let d be the number of duplicates in which no clones were observed and let n be the total number of DFP's scored. Then the probabilities of one or more clones occurring in each portion of a DFP are:

$$1 - (\frac{o}{n}) \quad \text{and} \quad 1 - (\frac{d}{n})$$

for the original and duplicate portions respectively. Multiplying these together gives the probability of one DFP having one or more clones in both the original and duplicate portions (I represent this probability as do).

$$\{1 - (\frac{o}{n})\}\{1 - (\frac{d}{n})\} = do$$

Multiplying do by the number of DFP's in that irradiation batch gives the expected number of such cases.

This number will, of course, be a maximum estimate as not all of the cases in which one or more clones occur in each portion of the DFP will resemble an OD clone. To do so both the O clone(s) and D clone(s) must mark structures at the border of the original and duplicate portions. Those DFP's in which two distinct clones could be scored, one in the original and one in the duplicate (called O + D) are shown separately from those in which one contiguous patch of marked tissue extended from the original to the duplicate. No attempt was made to remove possible spontaneous clones from the data sets. It was assumed that these clones would be small and unlikely to be confused with OD clones. Also, all treatment batches would have an equal frequency of spontaneous clones, so if spontaneous clones are occurring which resemble OD clones, all treatment batches would have them, i.e., there would be a constant "background" frequency of OD clones: Inspection of the data indicates this is not the case.

The expected number of OD clones for the 0-36 hour batches are shown in Table 29. At 0 and 12 hours after the 29° treatment substantially more than the expected numbers of OD plus O + D clones are observed. However, at 24 and 36 hours the expected number is greater or equal to the observed number, and by 48 hours no OD clones were observed. This lack of OD clones at 48 hours cannot be attributed to a decrease in the size of the clones. An example of a 48 hour D clone is shown in Figure 27. This clone marks a large area of this nearly complete duplicate, including the area along the original/duplicate border.

It is impossible, of course, to say whether any given clone is an OD or is two separate clones, however, these results suggest that no, or very few true OD clones are induced by irradiation given at 24 or more hours after the end of the 29° treatment. This result agrees well with the earlier, non-*Minute* clonal analysis and suggest that the original and duplicate portions of the DFP do become separate within 24 hours after the end of the 29° treatment.

Location of the Anterior/Posterior Compartment Boundary in Normal Legs

Before the duplicate portions of the DFP's could be scored for the presence of the anterior/posterior compartment boundary it was necessary to determine the location of this boundary in normal legs. To do this the clones in a sample of the 22° control legs from the irradiated 726 *Minute* females and males were scored for clone location. The boundaries of the marked trichomes and bristles of each clone were traced on standard diagrams of the mesothoracic leg. A sample of the clone boundaries are shown in Figure 28a traced on a single diagram.

In general the clones respect a boundary that agrees with that published by Steiner (1976). The tarsus, which was not shown on Steiner's

Figure 27 A photograph of a large D clone induced in a *726 Minute* male by irradiation at 36 hours after the end of the 29° treatment. The clone appears to respect the border between the original and the duplicate portions of the DFP. y = yellow bristle, + = non-yellow bristle, mwh = trichomes marked by multiple wing hairs

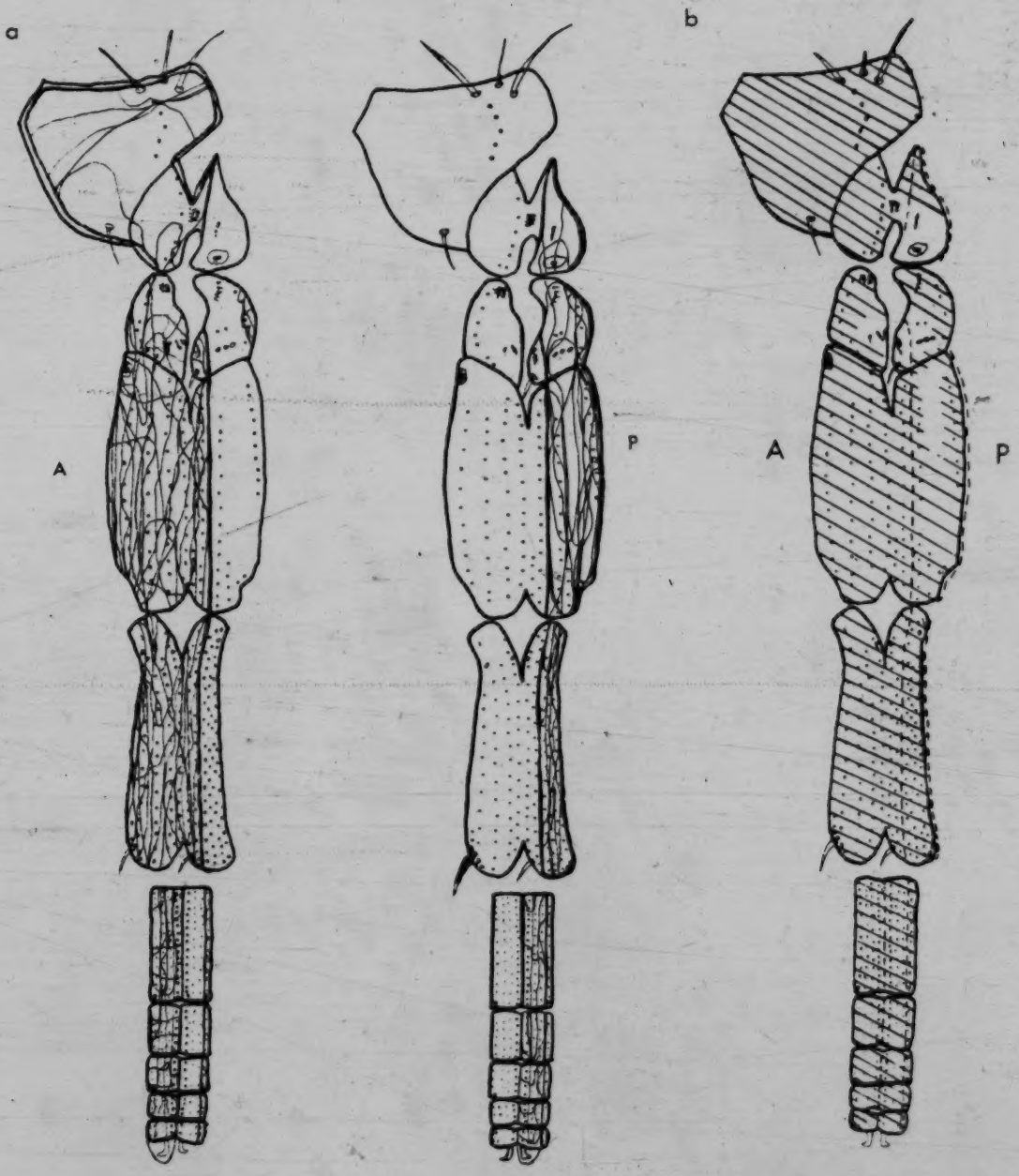


Figure 28 a Two diagrams showing the borders of clones in 22° control legs induced at 48 hours after the end of the midpoint of the egg collection period. The clones appear to respect an anterior/posterior boundary similar to that shown by Steiner (1976) with one exception noted in the text.
A = anterior clones, P = posterior clones

b The anterior/posterior boundary indicated by these clones drawn on the standard leg map.

maps, was asymmetrically divided by the boundary, with 5 bristle rows in the anterior and 3 in the posterior compartment (Figure 27b). One important exception was noted. Steiner's map indicates that the two rows of bristles in the trochanter are in separate compartments one in the anterior and one in the posterior. However, in the present study clones were found which appeared to mark both of these rows. Two examples are shown in Figure 29. In 28a, a portion of an anterior clone which appears to respect the boundary in the coxa and femur is shown marking the posterior row of bristles. In 29b, a portion of a posterior clone which also appears to respect the boundary elsewhere is shown marking this same row of bristles.

To investigate this situation further, the number of clones that appeared to respect the compartment boundary and marked these trochanter bristles was counted (Table 30). Three observations can be made from these data. First while anterior clones can mark posterior bristles, the reverse does not occur. Thus it is the "posterior" row of bristles that has a special status with respect to compartments. Second, approximately half (45%) of the anterior clones that approach this area mark the supposedly posterior bristles. Thirdly, when these posterior bristles are marked by a clone, the chances are roughly 3:2 that it will be a posterior clone.

A possibility that must be considered is that this situation is a result of cases in which two clones were induced independently, one an AR and the other a PR which are contiguous in this area of the trochanter by chance. This is not likely for two reasons. First, the small amount of tissue marked in the portion of the trochanter defined as posterior by Steiner implies that these two clones must differ

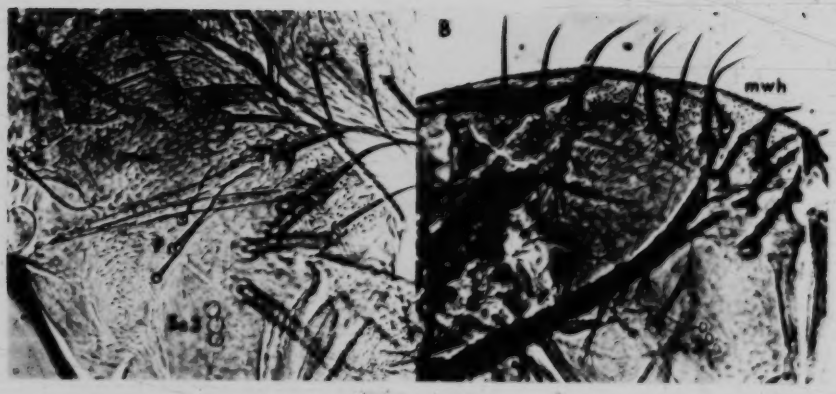


Figure 29 Photograph of two clones in 22° control legs which both mark the row of trochanter bristles shown as being in the posterior compartment of Steiner's map.
(a) A clone which is otherwise an AR clone
(b) A clone which is otherwise a PR clone.
y = yellow bristle + = non yellow bristle mwh = trichomes marked by multiple wing hairs

Table 30 The Number of Clones in each Compartment of 22° Control Legs which Respect the Boundary and Mark the Posterior Row of Bristles in the Trochanter[1]

Number of AR Clones Marking the Posterior Bristles	Number of AR Clones Marking only the Anterior Bristles	Number of PR Clones Marking the Anterior Bristles	Number of PR Clones Marking only the Posterior Bristles
13	16	0	21

1. AR=Anterior clones which otherwise respect the compartment boundary
PR=Posterior clones which otherwise respect the compartment boundary

greatly in size. This is not reasonable if both clones were supposedly induced by the same irradiation. If the PR is considered to be a small spontaneous clone, it is not likely that so many spontaneous clones (13 in 260 legs) would all be posterior, all be at the boundary, all be found in legs with another AR clone and all mark the same area of the trochanter.

Secondly, statistical calculations can be made to determine the number of expected legs in which two or more independent clones occurred in different compartments. The 13 cases considered in Table 30 were not the only cases in which a leg was found with both posterior and anterior compartments marked. In 12 other legs large areas of both compartments were marked. In 7 of these cases, however, the marked tissue was not contiguous and could be divided into an anterior and a posterior clone. Following the same method as was outlined above for the OD clones, the data was used to estimate the expected number of legs with one or more anterior and one or more posterior clones. In this calculation, the 12 cases with large anterior and posterior areas were considered to be two clones, while the 13 cases given in Table 30 were considered as single, anterior clones. With these assumptions there were 184 cases of anterior compartments with no clones and 213 cases of posterior compartments with no clones, in the 260 legs scored. Substituting these numbers into the equation described previously gives:

$$\{1 - (\frac{184}{260})\}\{1 - (\frac{213}{260})\}\ 260 = 13.8$$

Thus about 14 legs are expected to have both anterior and posterior clones. This is slightly more than the 12 cases observed, but is certainly not large enough to explain in addition the 13 cases of anterior

clones which mark posterior bristles in the trochanter.

Determination of the Anterior/Posterior Compartment Boundary in Duplicate Portions of DFP's

Following determination of the location of the compartment boundary in the 22° control legs, samples of D and OD clones were scored for evidence of a similar boundary in duplicates. First, the outline of each clone was traced on a standard leg diagram. A sample of the diagrams showing the location of D clones from the 48-72 hour irradiated batches in the *Minute* experiment were combined and are shown in Figure 30. The clones do not cross a line which appears to be identical to the anterior/posterior boundary defined in the 22° control legs. These results indicate that there is a compartment boundary in the duplicates which is the same as that seen in normal legs.

A question which naturally arises is: Does the boundary defined by the clones induced by late irradiation always exist, or does it arise at some time during the development of the duplicate? To answer this, the clones in each irradiation batch were classified according to their behavior with respect to this boundary. The clones marking trichomes and bristles in both compartments were designated *AP* clones. Only those cases in which a contiguous patch of marked trichomes and bristles was found which extended from the anterior to the posterior was scored as an AP clone. Those clones which intersected the boundary but failed to cross it were designated "respecting" clones, *AR* or *PR*, depending on whether they were in the anterior or posterior compartment. Those clones which failed to approach the boundary were simply designated A or P clones, for anterior or posterior respectively.

In addition, samples of the twin spots from the previous, non-*Minute* clonal analysis were scored and classified in the same way. It

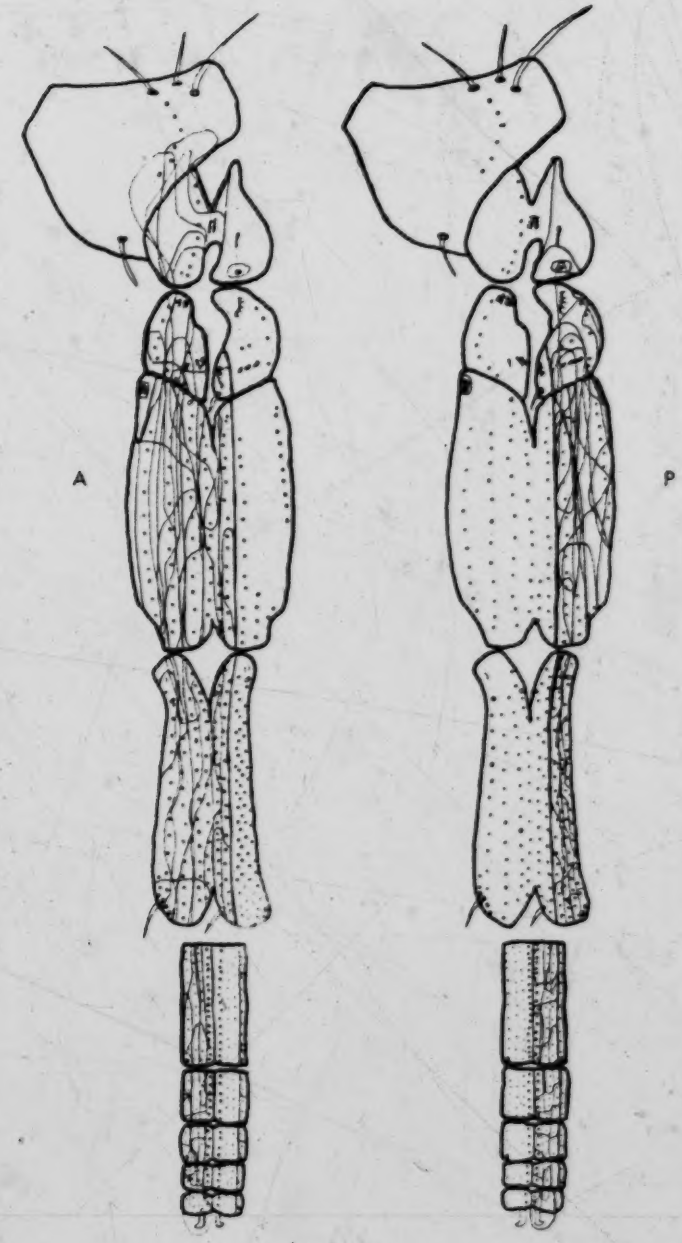


Figure 30 Two diagrams showing the outlines of all D clones induced by radiation given 48 hours or more after the end of the 29° treatment. The clones appear to respect a boundary identical to that in the 22° control legs.
A = anterior clones, P = posterior clones

be noted that the two spots of a twin were scored as one clone, eg., if one of the spots crossed the boundary the clone was scored as being an AP clone.

Following this classification the number of each type of clone in each irradiation batch was counted. First, in the non-*Minute* experiment, clones from batches irradiated prior to the end of the 29° treatment 19 out of 75 clones were found to be AP clones and no AR or PR clones were found. An example of such an AP clone is shown in Figure 31. This clone marks almost the entire duplicate. This suggests that the boundary is formed at some time during the development of the duplicate. To determine when this occurs, the kinds of clones initiated at and after the end of the 29° treatment were examined in both the non-*Minute* and *Minute* experiments.

The numbers of clones of each type (AP, AR, PR, A and P) in samples taken from the non-*Minute* experiment are given in Table 31. At 0 hours (at the end of the 29° treatment) only AP, A and P clones were found. At 24 and 48 hours, however, AR and PR clones were also found. Clones induced at 72 hours were too small to reliably classify. These results suggest that the compartment boundary is formed between 24 and 48 hours after the end of the 29° treatment.

Three interesting clones were found in the 24-48 hour batches which were twin spots with one spot in each compartment. In each of these cases the *mwh* sn^3 spot could be seen to respect the boundary. These clones indicate that the boundary is established within the space of a single cell division in an individual duplicate. There does, however, appear to be some variation between duplicates as to when this boundary is established.

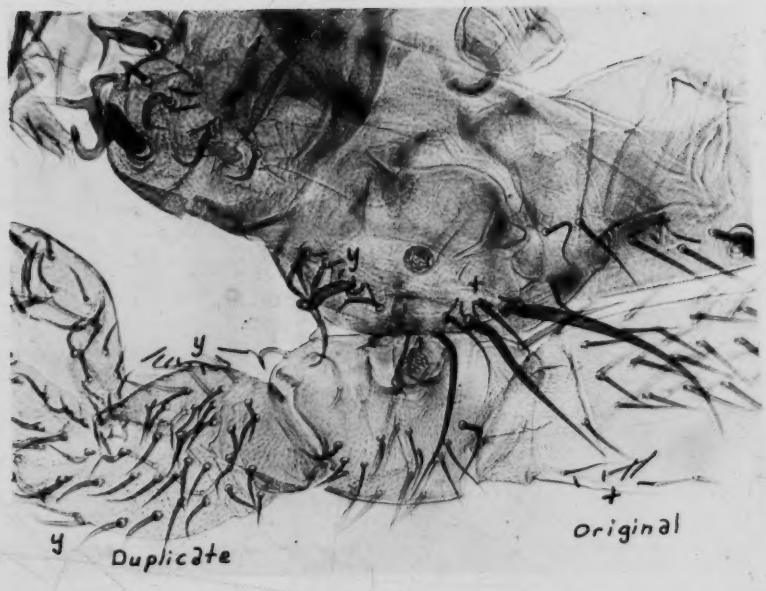


Figure 31 A photograph of a very large OD clone induced in a *726* female by irradiation prior to the end of the 29° treatment. This clone marks structures in both the anterior and posterior of the duplicate.
y = yellow, forked bristles + = wild type bristles

Table 31 The Subdivision of D Clones into A, P, AP, AR and PR Types in 726 Females Irradiated at or after the end of the 29° Treatment[2]

Developmental Stage at Irradiation [1]	DFP #	Clones #	Clones %	Clone Types A	P	AP	AR	PR
0	232	31	13	15	10	6	0	0
24	260	40	15	22	9	1	2*	2
48	246	63	26	34	19	1	5	4**
72	72	51	71	36	15	0	0	0

1. Given as hours after the end of the 29° treatment

* Includes 1 leg with *yf* posterior and *mwh* sn^3 AR clone

** Includes 2 legs with *yf* anterior and *mwh* sn^3 PR clones

2. See text for a description of the clone types

A question which arises here is, how many cells form the observed anterior and posterior compartments of the duplicate? Estimates of the total cell number in the duplicate have been made (6.7 and 21) and the clone frequency data can be used to divide these into anterior and posterior portions. To do this the percentage of the clones marking only the anterior compartment in the duplicate was calculated for each irradiation batch (Table 32). For comparison, the same frequencies for the clones in the 22° control legs, the 29° treated control legs and the original portions of the DFPs are also given in Table 32. The frequency in all of the legs is about 66%, and no obvious trend is noted from batch to batch. Thus the relative proportion of anterior cells appears to remain the same throughout the development of the duplicate, and it appears to be similar to that of normal legs. Multiplication of the total number of cells in the duplicate by the fraction of anterior clones gives estimates of 4.5 and 14 cells in the anterior compartment at the time of initiation of the duplicate, using the mean clone size and smallest clone size estimates, respectively. By subtraction, 2.2 and 7 cells are estimated to be in the posterior compartment at the same time.

The data from the non-*Minute* experiment were not extensive enough to precisely define the time of appearance of the compartment boundary in duplicates. As a further investigation of this point samples of the clones in duplicates from 726 *Minute* females and males were examined. The frequency of the clones in these samples and the number in each classification are given in Table 33. No AR or PR clones were observed in the 0 or 12 hour batches, and no AP clones were observed in the 48-72 hour batches. A portion of two examples, AP and a PR clone are shown in Figure 32.

Table 32 The Percentage of Clones in *726* Female DFPs which Mark Only the Anterior Compartment in *726* Females

Age at Irradiation	Percentage of Clones			
	22° Control	29° Treated Control	Original Portions	Duplicate Portions
24	67.6	61.9	70.6	68.8
48	55.6	77.8	76.5	64.3
96	69.0	62.5	67.9	64.2
120	64.6	75.0	58.8	63.6
144	66.7	70.0	65.9	60.9
168	72.2	66.7	72.3	59.1
192	61.0	69.8	65.0	73.9
216	58.2	67.3	65.2	62.1
Overall average	69.3	70.7	66.9	66.3

Table 33 The Subdivision of D Clones into A, P, AP, AR and PR Types in 726 *Minute* Adults irradiated at or After the end of the 29° Treatment

Developmental Stage at Irradiation[1]	DFP	Clones		Clone Types				
	#	#	%	A	P	AP	AR	PR
0	81	24	30	10	4	10	0	0
12	150	42	28	23	7	12	0	0
24	86	26	30	15	7	3	0	1
36	58	21	36	12	3	2	1	3
48	22	16	73	9	4	0	2	1
60	25	23	92	14	4	0	2	3
72	19	24	126	13	6	0	4	1

1. Given as hours after the end of the 29° treatment

2. See text for a description of the clone types (p.142)

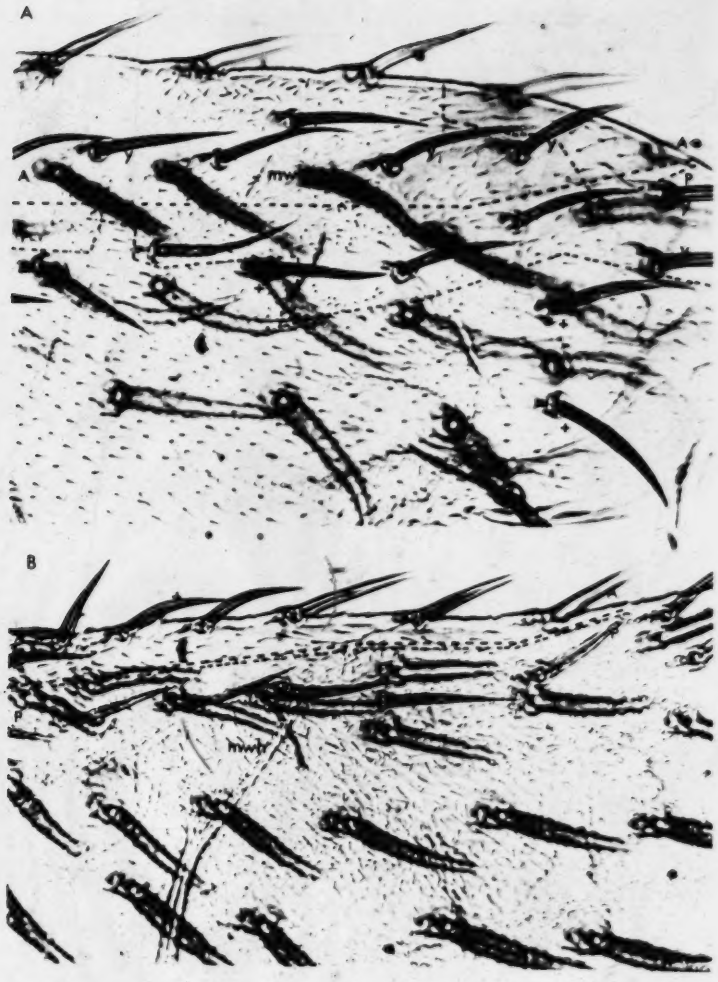


Figure 32 Photographs of two D clones in *726 Minute* females which mark the femur. (B) A PR clone which respects the boundary (A) An AP clone which crosses the boundary. The area marked by the clones is outlined.
y = yellow bristle + = wild type bristle mwh = trichomes marked by multiple wing hairs

A question which arises at this point is: Are the AP clones observed actually two independent clones, one an AR and the other a PR, which happen to occur in the same duplicate? This is not likely as these batches contain several AP clones and no AR or PR clones. It is highly unlikely that all AR and PR clones in these batches occurred in matched pairs. In addition to this consideration, however, statistical calculations were done on the data from each irradiation batch to determine the expected number of AP clones produced by chance alone. The calculations were the same as those described previously in the investigation of the OD clones in DFP's. In each calculation all AP clones were assumed to be two clones, one AR and one PR. The probability of two or more independent clones occurring in one duplicate was calculated as before, but an additional factor was added. Unlike the large clones induced in the 22° control legs, many of the clones in the duplicates fail to approach the compartment boundary. This is due to the decrease in clone size that occurs with later irradiation. Both of the clones must intersect the boundary before they can be mistaken for a single AP clone. Thus the probability that both of the clones will approach the compartment boundary is included in the calculation.

The calculation used and the resulting expected number of AP clones for the 0-36 hour batches are shown in Table 34. Very few apparent AP clones are expected by chance. This confirms that the observed AP clones really are single clones which do not respect the compartment boundary. Thus these results suggest that the anterior/posterior compartment boundary is established in duplicates within 48 hours after the end of the 29° treatment. It is worth noting that the original/duplicate distinction is established earlier, by 24 hours, at or just before the duplicates begin to grow (at 48 hours as indicated by the increase in clone frequency).

Table 34 The Expected Number of Cases in Which Two or More Independent Clones Might Mimic an AP Clone[1]

Developmental Stage at Irradiation (hr)	T	a	p	AR	PR	Number of AP Clones Observed	ap
0	81	61	67	10	10	10	1.20
12	150	115	131	12	12	12	.93
24	86	60	75	3	4	3	.14
36	58	43	50	3	5	2	.26

1. Calculated as:

$$\left(1 - \frac{a}{T}\right)\left(1 - \frac{p}{T}\right)\left(\frac{AR}{T - a}\right)\left(\frac{PR}{T - p}\right) \times T = ap$$

where a = The number of Anterior compartments with no clone

p = The number of Posterior compartments with no clone

T = The number of duplicates scored in that batch

AR = The number of Anterior clones that respect the boundary

PR = The number of Posterior clones that respect the boundary

ap = The expected number of duplicates in which an AR and a PR clone might mimic an AP clone

DISCUSSION

The implications of the *ts726* DFP phenotype in relation to current models of pattern formation

One of the main purposes of the research reported in this thesis was to investigate the phenotype of *ts726* induced DFP's, and to relate the findings to current models of pattern formation. Two observations can be made from the results of this analysis. First, the structures in the medial portion of the disc are often deficient and are rarely duplicated, while lateral structures are rarely deficient and are often duplicated. This is analogous to the pattern of deficiency and duplication observed in cultured leg disc fragments (Schubiger 1971, Strub 1977 a,b). Second, the deficiencies in the DFP's often include only a few markers, and in a sizable fraction of cases (56/893) do not include any at all. This is different from the disc fragment results in which discs with a small surgically induced deficiency in the upper medial quarter of the disc do not duplicate.

These two observations can be related to the polar coordinate model of French et al. (1976) which was designed to account for the results of fragmentation experiments done on the first leg disc. This model proposes that the pattern of deficiency/duplication seen in the DFP's is a consequense of there being a higher concentration of circular positional values in the upper medial quarter of the disc. Whenever a patch of cell death removes enough of the cells in this region to eliminate half of these values, the confrontation of surviving cells at the edges of the death patch would initiate a duplication. The second observation is at odds with the model. The observed small size of the deficiencies associated with duplications suggests that one half of the circular values are

compressed into a very small portion of the upper medial quarter of the disc. These values must be so compressed that even a patch of *ts726* induced cell death that does not affect a single marker can remove over half of the values.

There are several alternative explanations for this aspect of the DFP phenotype. First, it is possible that cell death is occasionally produced in long, thin patches in the mesothoracic leg discs, such that much of the edge of the upper medial quarter is removed without affecting many of the morphological markers (see Postlethwait 1978). This is not considered likely as earlier histological studies of 29° treated *ts726* imaginal discs reveal no such patterns of cell death (Clark 1976, Clark and Russell 1977). The high frequency of small deficiencies (359/893 affect one or no markers) implies that such patches should have been found in even a small sample of leg discs.

Another alternative is that the organization of the circular values in the mesothoracic leg disc is different from that in the prothoracic leg disc and that, the values are not more concentrated in the upper medial quarter of the disc. In the present study large regions of the upper leg segments had very few morphological markers. As shown in Figure 5 a large portion of the thorax and femur are unmarked in the lower medial quarter of the disc (eg., between ST and St4). Since St4 is very often deficient, it is possible that the patches of cell death are removing enough circular markers in the lower medial quarter to induce a duplication. This is supported by the observation that in two DFP's the St4 is deficient and the ST is not. There is firm evidence for the concentration of circular values in the prothoracic leg into the upper medial quarter of the disc, but no such evidence has been published for the mesothoracic leg disc. However, in a recent communication, Schubiger

(unpublished) suggests that the circular values in the mesothoracic leg disc are uniformly distributed around the disc circumference, supporting this alternative explanation.

A third alternative explanation is that the effects of removing cells by a clean cut and by a patch of cell death are not the same. It is possible that a patch of dead cells might interfere with the wound healing process necessary for intercalation. In a preliminary test of this possibility a series of discs with small, surgical deficiencies for the areas most often deficient were cultured *in vivo* for 5 days. No cases of duplication were observed. This result is compatible with either this or the previous explanation, and only further work will allow the two to be distinguished.

One final possibility is that following the induction of cell death the imaginal disc first regenerates and then duplicates. This would imply that the observed deficiency is not a valid indicator of the size of the patch of cell death. This is not compatible with the polar coordinate model. According to the model regeneration and duplication are mutually exclusive events. If a majority of the circular values are removed intercalation must produce only a duplication. No mechanism exists for regeneration in such a fragment. This possibility is not considered likely, due to the observations made in the clonal analysis of duplications. If regeneration and duplication were occurring then a large number of OD clones should be produced by radiation treatments given at or shortly after the end of the 29^{o} treatment. This would be due to the initial marked cells of the clone participating in both the regeneration and the duplication. This is not observed. The number of OD clones is very low in batches irradiated during or shortly after the 29^{o} treatment.

Of the alternative explanations presented above, the possibility that the organization of the positional values is different in the mesothoracic and prothoracic legs is favored here. This explains the observation, and the high frequencies of deficiency of markers 2St6 and St4, both of which are in the lower medial quarter of the disc. This explanation is also reasonable in view of the organization of the coordinate systems proposed by French et al. for the amphibian and the cockroach limb.

The Determinative Events in the Development of Duplicates

In the introduction it was proposed that in *Drosophila*, cells become sequentially determined to form specific adult structures. At each step in this process cells are proposed to decide between alternative developmental pathways on the basis of their positional information. In order to explain the processes of regeneration and duplication it was also proposed that these processes are reiterations of normal development undertaken by cells which have "forgotten" certain of the sequential decisions made by their ancestors. The second main purpose of the present work was to test this hypothesis by investigating the intermediate stages of development of duplications using the technique of clonal analysis. Two aspects of development were chosen for study. First, the number of cells present in duplicates at several stages was estimated and second, the locations of clones induced in duplicates at different times after the 29° treatment were examined for evidence of the existence of an anterior/posterior compartment boundary. The *Minute* technique was used in this second analysis.

The question of the number of cells in the duplicate has been previously investigated in cultured disc fragments using a radioactive cell marker (Wildermuth 1968 a,b) and clonal analysis (Ulrich 1971, Postlethwait et al. 1971, Nöthinger 1976). However, the limitations of the transplantation technique for the generation of large numbers of cases and the restriction of these investigations to marking cells at only one time point prevented any detailed conclusions. The large numbers of cases and multiple irradiation times in the present study allow a more detailed comparison of normal and duplicate growth.

The number of cells present in the duplicate was estimated in two ways. The first of these uses half of the inverse of the mean relative

clone size as an estimate of the number of cells present at the time of irradiation (Becker 1957; Bryant and Schneiderman 1969, Garcia-Bellido and Merriam 1971; Wieschaus and Gehring 1976a). This method assumes that the ratio of marked to unmarked cells at the time of segregation of the clone is maintained throughout development. The inverse of this ratio is thus an estimate of the number of cells present at the time of segregation of the first marked cell, and half of this estimates the number at the time of irradiation.

Using this method, estimates of the number of cells in the normal leg in the embryo have been made (Bryant and Schneiderman 1969; Wieschaus and Gehring 1976). These estimates range from 7-10 cells, based on clones marking leg bristles. In the current experiment, the earliest irradiation time was 24 hours after the midpoint of the egg laying period and the estimate of the number of cells in the control legs at this time was about 15. This is higher than the estimates of cell number in the embryo, which is likely due to the fact that most of the subjects were beyond the embryo stage at the time of irradiation. Applying this method to duplicate clones initiated by the irradiation given 24 hours after the end of the 29° treatment, i.e., just prior to the time at which the duplicates begin to grow, gives an estimate of 6.7 cells. This number is very close to the number estimated for normal legs in the embryo.

A second method has been used to determine the number of cells which become committed to form an imaginal disc. In this method mosaics are generated before the disc is initiated (usually gynandromorphs are used) and the smallest mosaic patch observed is assumed to indicate the case in which only one of the initiating cells was of a different genotype (see Wieschaus and Gehring 1976b and Madhavan and Schneiderman 1977 for review). The ratio of unmarked to marked tissue in the smallest

clone is assumed to represent the number of cells present at initiation. Being based on only one clone in each treatment batch this estimate cannot be considered to be very accurate. Variations in the local growth rate within the imaginal disc will tend to reduce the smallest clone size and hence gave an overestimate of the cell number.

In previous studies in which gynandromorphs were used the number of cells which initiate the leg disc was found to be about 20 (Madhavan and Schneiderman 1977 for review of these studies). To make a comparable estimate of the number of cells initiating the duplicate in the present study the smallest clone from each irradiation batch was examined. The estimates were remarkably constant in all batches irradiated before the end of the 29^{o} treatment, averaging 21 cells. Thus this second estimate of the number of cells in the duplicate at initiation is very close to estimates of the number of cells initiating the normal leg. These two comparisons lend support to the hypothesis that the duplicate leg develops by a reiteration of normal developmental processes.

The rate of growth in imaginal discs has been estimated from the rates of change of the frequency and size of clones induced throughout development (Becker 1957; Bryant and Schneiderman 1969; Garcia-Bellido and Merriam 1971). In these studies the logarithms of clone frequency increased and those of the clone size decreased linearly with time of development, indicating that the imaginal discs were an exponentially growing population of cells. The slopes of the lines fitted to these logarithms were used to estimate the rate of growth of the discs. In the leg this rate, expressed as the number of hours required for a doubling of the clone frequency or halving of the clone size, was about 15 hours at 25^{o} (Bryant and Schneiderman 1969). The same calculations on the control legs grown at 22^{o} in the present study gave estimates of

19.6 and 20.0 hours (from the frequency and size curves respectively), a reasonable agreement with the previous estimate.

In the 29° treated control legs and the DFP's the slopes of these curves are not constant. During and for about 24 hours after the 29° treatment the clone frequency does not change very much in the 29° control legs. Since cell death is known to occur during this period, this implies that the rate of residual cell division at 29° is approximately equal to the rate of cell death.

The rates of growth appear to return to normal in the 29° treated control legs and in the original portions of DFP's about 24 hours after the return to 22°. The slopes of the clone frequency and clone size curves are not significantly different from those of the 22° control legs after this point. This implies that the cells have recovered from the treatment and have resumed normal growth. The slopes of the lines fitted to the duplicate portions of DFP's during this period were not significantly different from those of the original portions of the DFP's. This implies that once growth has begun in the duplicates it proceeds at about the normal rate. Thus the calculations of rate of growth also support the hypothesis that the duplicates are developing via a reiteration of normal development.

One additional event which is known to occur in normal development is the establishment of a compartment boundary separating the cells forming the anterior and posterior structures. In the mesothoracic leg firm evidence exists for only a single anterior/posterior compartment boundary (Steiner 1976). This boundary appears to arise at or shortly after the cellular blastoderm stage.

If compartmentalization events reflect determination events occurring in groups of cells throughout development and, if pattern duplications

truly reiterate normal development, an anterior/posterior boundary should appear in the duplicate portions of DFP's. If, on the other hand, these boundaries are simply an indication of passive segregations of cells then no boundary or a boundary in a different location might be expected in the duplicates. Such a result would argue against compartments being important in determination as it would indicate that there are mechanisms for growing a leg without compartmentalization. Alternatively, the appearance of a compartment boundary in the same location as in normal legs would provide evidence for both the developmental significance of compartmentalization and for the hypothesis of duplication as a reiteration of normal development.

As a first step in the analysis of the *ts726* induced leg duplications for compartment boundaries, the location of the boundary in 22° control legs was determined. The results confirmed the findings of Steiner (1976) with one notable exception. One row of bristles in the trochanter appeared to be marked by clones from both compartments. These bristles were placed in the posterior compartment by Steiner, but a large number of anterior clones marked them. These clones were too numerous to be cases of two independent clones.

Several possible reasons can be considered for the behavior of these clones. First, Steiner's map might be incorrect, and this row of bristles might be actually in the anterior compartment. This is not tenable as many posterior clones which respect the boundary elsewhere also mark these bristles. Also, anterior clones which mark the boundary elsewhere do not mark this row of bristles uniformly. Some mark only 1 bristle, some another, and some several bristles. There is no pattern which suggests a uniform boundary near or among the bristles.

Secondly, there might actually be no boundary in the trochanter. This is not tenable as the large anterior clones do not mark more than these few bristles and as posterior clones do not mark anterior structures. Were there no restrictions, these clones should mark large portions of both compartments in the trochanter. Also, these anterior clones do appear to respect the other anterior/posterior compartment boundary in the trochanter. This boundary can be precisely located in the patch of trichomes close to the edge bristle.

Thirdly, the compartment boundary might vary in location from leg to leg in this region of the trochanter such that these bristles are sometimes anterior and sometimes posterior. This can explain the data, but if it is true, then an important question is raised as to the function of compartments in development. It suggests that either both compartments might have the capacity to form these bristles, or that perhaps the compartments are passive divisions of cells which receive further instructions as to what structures to form from some other system of information.

The fourth possibility is that the irradiation used to induce the clone is inducing enough cell death to occasionally allow a marked cell to regenerate across the compartment boundary. The work of Schubiger (1971) has demonstrated that anterior cells can regenerate posterior structures, but that the reverse does not occur. This possibility would explain the limited extent of the anterior clones' penetration into the posterior compartment and the failure of posterior clones to invade the anterior compartment. The reason why this invasion should only occur in the trochanter is, however, not clear.

Of these possibilities the fourth, that the irradiation used to induce the mitotic recombination is inducing enough cell death to cause regeneration across the compartment boundary is favored here. It is known

that 1500 r causes considerable cell death in the imaginal disc (50-60% according to Haynie and Bryant 1976). By random chance this death might occasionally produce a small "hole" in the disc that removes cells at the compartment boundary. Since anterior cells can regenerate posterior structures, but not the reverse, this could explain why anterior clones occasionally mark a small portion of the posterior structures.

Following the investigation of the compartment boundary in normal legs, clones induced in duplicates were examined. Three observations were made from the results. First, clones induced by a late irradiation were restricted by a boundary. Secondly, this boundary appeared to be in exactly the same location as the anterior/posterior boundary described in the normal legs. Thirdly, analysis of clones induced at different times indicated that this boundary appears in the duplicate between 24 and 48 hours after the end of the 29° treatment.

These observations provide strong evidence for the hypothesis that compartmentalization events are important in the development of imaginal discs and for the gorwth of duplicates being a reiteration of normal development. The appearance of an apparently normal compartment boundary in the early stages of duplicate development under conditions that are likely to be very different from those in the embryo implies that the establishment of this boundary is an inherent part of the mechanism for generating leg structures.

Thus both aspects of duplicate growth examined by clonal analysis are very similar to the growth of normal legs. This supports the hypothesis that cells become determined by a series of sequential steps under the control of positional information and that duplication occurs by a reiteration of some of these steps. The observations made on duplicative growth also suggest that the decisions are not forgotten at random

at the initiation of a duplication. The anterior/posterior compartment boundary is established prior to the committment to form a specific disc, yet the decision to form a disc is remembered in culture while the compartmentalization is not. This suggests that the sequential determinative events have different stabilities.

One possible reason for this difference might be that those decisions which must be reiterated during regeneration have a low stability. This is supported by the current observations. The decision to form a particular part of the disc must be reiterated during regeneration and has a low stability while the decision to form a particular imaginal disc must be remembered and has a high stability. It thus seems reasonable to conclude that the stability of the determinative decisions is directed towards ensuring that regeneration produces a correct copy of the missing structures.

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APPENDIX A The Number of Elements Present in each Marker in the Imaginal Disc Fragments Transplanted into Mature Larval Hosts

Cut		ThB	Sc'5	St1	Sc1	St8	ST	1St7	2St7	2St6	2St6	Tsp	AB	Tsc3	TST	mSc1	St4	St5	Sc11	EH'	Sc1	Sc'8	EB	TSc2	PAB	SP	mSc2	3Sc2	Un	Unp	Em	AP
1A	1	3	5	1	1	8	1	3	4	3	3	4	1	3	3	1	4	3	6	1	0	0	0	0	0	0	0	0	0	0	0	0
	2	1	3	1	0	8	1	3	4	3	3	3	1	3	2	0	4	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	3	5	1	1	8	1	3	4	3	3	5	1	3	3	0	4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	2	5	1	1	8	1	3	4	3	3	3	1	2	2	1	4	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	1	2	0	0	4	1	3	4	3	3	1	0	2	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
1B	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	8	1	2	1	2	1	1	2	2	1	1
	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	1	3	8	1	2	0	1	0	0	0	0	0	0
	3	2	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	3	5	1	3	8	1	2	1	2	2	2	2	2	1	1
	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	1	3	8	1	2	1	2	0	1	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	8	1	3	8	1	2	1	2	1	2	2	2	1	1
	6	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	2	1	2	2	2	2	2	1	1
2A	1	6	5	1	1	8	1	3	4	3	3	3	1	1	0	0	0	0	0	0	0	4	1	1	1	2	1	1	2	2	1	1
	2	5	5	1	1	8	1	3	4	3	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0
	3	6	4	1	1	8	1	3	4	3	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0
	4	6	5	1	1	8	1	3	4	3	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
	5	6	5	1	1	8	1	3	4	3	2	3	1	2	0	0	0	0	0	0	0	0	0	0	0	2	1	1	0	0	0	0
	6	6	6	1	1	8	1	3	4	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
2B	1	0	0	0	0	0	0	0	0	0	0	2	0	2	3	1	4	5	11	1	3	8	1	1	1	0	1	1	2	2	1	1
	2	0	0	0	0	0	0	0	0	0	0	1	0	1	3	1	4	5	6	0	0	6	0	0	0	0	0	0	1	0	1	1
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	4	5	10	1	3	4	0	0	0	0	0	0	2	1	1	1
	4	0	0	0	0	0	0	0	0	0	0	4	1	3	3	1	4	5	11	1	3	8	1	2	1	0	0	1	2	2	1	1
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	5	11	1	3	6	0	0	0	0	0	0	1	1	1	0
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	4	5	4	0	0	8	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0	1	0	3	2	1	4	5	11	1	3	8	1	0	0	0	0	0	2	2	1	1
	8	0	0	0	0	0	0	0	0	0	0	3	1	3	2	0	4	5	10	1	3	6	0	0	0	0	0	0	1	2	1	1
5A	1	6	5	1	1	8	1	3	4	1	0	0	0	0	0	0	0	0	0	0	0	8	1	2	1	2	2	1	2	2	1	1
	2	6	5	1	1	8	1	3	4	2	0	2	0	0	0	0	0	0	0	0	0	8	1	2	1	2	2	0	2	2	1	1
	3	6	5	1	1	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	2	1	2	1	0	0	0	0	0
	4	6	5	1	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
	5	6	5	1	1	8	1	3	1	0	0	0	0	0	0	0	0	0	0	0	0	3	0	1	0	2	1	1	2	2	1	1
	6	6	5	1	1	8	1	3	2	0	0	0	0	0	0	0	0	0	0	0	0	8	1	2	1	2	0	0	2	2	1	1
	7	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
3B	1	0	0	0	0	0	0	0	0	0	1	3	0	0	0	0	0	4	5	11	1	3	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	3	3	4	1	3	4	1	4	5	11	1	3	4	0	0	0	0	0	1	2	2	1	1	1
	3	0	0	0	0	0	0	0	0	2	3	3	5	1	3	4	1	4	5	11	1	3	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	2	2	0	1	3	1	4	5	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4A	1	5	5	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3	8	1	2	1	2	2	1	2	2	1	1	1
	2	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	2	0	0	0	0	0	0	0
	3	6	5	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	8	1	2	1	2	1	2	2	2	1	1	1
	4	6	5	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	1	2	1	2	1	0	2	2	1	1	1
4B	1	0	1	0	0	8	1	3	4	3	3	5	1	3	2	1	4	5	10	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	1	2	1	0	8	1	3	4	3	3	5	1	3	3	1	4	5	11	1	3	0	0	0	0	0	0	1	2	2	1	1	1
	3	0	0	0	0	3	1	3	4	3	3	0	0	0	0	0	4	3	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	2	1	0	8	1	3	4	3	3	4	1	3	3	1	4	5	11	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	6	1	3	4	3	3	5	1	3	3	1	4	5	11	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	2	3	3	5	1	3	3	0	4	5	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

APPENDIX B. Organization of the data on computer cards and computer programs used

1. Organization of the morphological marker data on computer cards

Columns[1]	Data
1-6	card identity number
8-10	experiment number
12-14	larval age at 29° treatment (hours)[2]
16-17	date of eclosion (days)[3]
19	leg type[4]
21	ST[6]
22	SP
23-24	ThB
25	status of thorax[5]
25	St4
27-28	St8
29	BH^-
30	status of coxa[5]
31	St7
32	St6
33	St5
34	Sc3
35	St1
36	EB
37	Sc^+5
38-39	Sc^-8
40	status of trochanter[5]
41-42	Sc11
43	Sc1
44	status of femur[5]
45	TST
46	TSc2
47	TSc3
48	PAB
49	AB
50	Tsp
51	status of tibia[5]

APPENDIX B No. 1 (cont'd)

52-54	number of bracted tarsal bristles
55	mSc1
56	mSc2
57	3Sc2
58	number of adventitious bristles
59	number of segments in tarsus
60	status of tarsus[5]
61	Un
62	Em
63	P
64	Unp
65	Ap
66	status of claw organ[5]
68	scoring procedure[7]
70-75	number of legs with these identical data

1. Columns not mentioned were left blank.

2. Recorded as hours after the midpoint of the egg collection.

3. Given as day on which eclosion occurred, 99=pharate adult

4. 1=Original portion 2=Duplicate portion 3=29° treated control
   4=22° control

5. Indicates whether the two portions of the DFP are separate
   1=Not separate 2=Partially separate 3=Completely separate

6. Given as the number of elements present
   -=Not scorable

7. 1=Completely scored 2=Tarsal bristles not scored 3=Scored on the index card

2. Examples of the data on computer cards

```
45492 285  90 01 1 022022071276431150821113423115315512205321211 3  1
45492 285  90 01 2 021221071276431030021002201003201000001201110 2  1
45582 285  90 01 1 022024071276531150831113423115315512205321211 3  1
45582 285  90 01 2 021223061254300000220802203004205510001201210 2  1
45612 285  90 01 1 022024091276531150831113423115315512205321211 3  1
45612 285  90 01 2 020524071250000000020001000000100000000010000 1  1
45632 285  90 02 1 022020070276531150831113423115315512205321211 3  1
45632 285  90 02 2 001020060276300000020712303005200500001200000 1  1
```

3. Recoding program run on raw data

```
C INITIAL RECODE PROGRAM

  VARIABLE LIST   ID,EXP,HOUR,ECLSN,PS,P1 TO P31,S1 TO S31
  INPUT MEDIUM    DISK
  N OF CASES      893
  INPUT FORMAT    FIXED(F6.0,1X,F3.0,1X,F3.0,1X,F2.0,1X,F1.0,1X,
                  2A1,A2,1X,A1,A2,A1,1X,7A1,
                  A2,1X,A2,A1,1X,6A1,1X,3A1,
                  3X,5A1,/,
                  20X,2A1,A2,1X,A1,A2,A1,1X,7A1,
                  A2,1X,A2,A1,1X,6A1,1X,A3,3A1,3X,5A1)
  RECODE          P23,S23('---'=-1) (CONVERT)/
                  P3,S3,P5,S5,P14,S14,P15,S15('---'=-1) (CONVEPT)/
                  P1,S1,P2,S2,P4,S4,P5,S5,P6 TO P13,S6 TO S13,P16 TO P22,
                  S16 TO S22,P24 TO P31,S24 TO S31('---'=-1) (CONVERT)
  COMPUTE         P32=4
  COMPUTE          P33=P7-4
  COMPUTE         P34=4
  COMPUTE         P35=P8-4
  IF              (P7 LT 4)P32=P7
  IF              (P7 LT 4)P33=0
  IF              (P8 LT 4)P34=P8
  IF              (P8 LT 4)P35=0
  COMPUTE         S32=4
  COMPUTE         S33=S7-4
  COMPUTE         S34=4
  COMPUTE         S35=S8-4
  IF              (S7 LT 4)S32=S7
  IF              (S7 LT 4)S33=0
  IF              (S8 LT 4)S34=S8
  IF              (S8 LT 4)S35=0
  IF              (P7 EQ -1)P33=-1
  IF              (P8 EQ -1)P35=-1
  IF              (S7 EQ -1)S35=-1
  IF              (S8 EQ -1)S35=-1
  RECODE          P1,S1(1=2) (3 THRU HIGHEST=4)/
                  P2,S2(3=2) (5 THRU HIGHEST=5)/
                  P3,S3(1 THRU 14=1) (15 THRU 26=2) (27 THRU HIGHEST=4)/
                  P4,S4(2,3=1) (4=2) (5 THRU HIGHEST=4)/
                  P5,S5(2 THRU 7=1) (8=2) (9 THRU HIGHEST=4)/
                  P6,S6(1=2)(2 THRU HIGHEST=4)
  RECODE          P7,S7(2,3,4,5,6=1) (7=2) (8 THRU HIGHEST=4)/
                  P8,S8(2,3,4,5=1) (6=2) (7 THRU HIGHEST=4)/
                  P9,S9,P13,S13(2,3,4=1) (5=2) (6 THRU HIGHEST=4)/
                  P14,S14(2 THRU 7=1) (8=2) (9 THRU HIGHEST=4)/
                  P15,S15(2THRU 10=1) (11,12=2) (13 THRU HIGHEST=4)/
                  P17,S17(2=1)(3,4=2) (5 THRU HIGHEST=4)
  RECODE          P23,S23(2 THRU 137=1) (138 THRU 173=2)
                  (174 THRU HIGHEST=4)/
                  P22,S22(2,3=1) (4,5,6=2) (7 THRU HIGHEST=4)/
                  P11,S11,P12,S12,P16,S16,P20,S20,P21,S21,
                  P24,S24,P26,S28,P30,S30,P31,S31(1=2) (2THRU HIGHEST=4)
  RECODE          P10,S10,P25,S25,P26,S26,P27,S27,P29,S29,P35,S35
                  (3 THRU HIGHEST=4)/
                  P32,S32,P34,S34(2,3=1) (4=2) (5 THRU HIGHEST=4)/
                  P10,S10,P19,S19,P33,S33(2=1) (3=2) (5 THRU HIGHEST=4)
  RECODE          ALL (-1=9)
  VAR LABLES      P1,STERNAL BRISTLE/P2,STERNOPLURAL BRISTLES/
                  P3,NO. OF THORACIC BRISTLES/
                  P4,GROUP OF 4 SENSILLA TRICHODEA/
                  P5,GROU  OF 8 SENSILLA TRICHODEA/
                  P6,HAIRY ISLAND BRISTLE/
                  P6,GROUP OF 7 SENSILLA TRICHODEA/
                  P8,GROUP OF 6 SENSILLA TRICHODEA/
                  P9,GROUP OF 5 SENSILLA TRICHODEA/
                  P10,GPOUP OF 3 SENSILLA CAMPANIFORMIA/
                  P11,GROUP OF 1 SENSILLA TRICHODEA/
                  P12,EDGE BRISTLE/
                  P13,GROUP OF 5 SENSILLA CAMPANIFORMIA/
                  P14,GPOUP OF 8 SENSILLA CAMPANIFORMIA/
                  P15,GROUP OF 11 SENSILLA CAMPANIFORMIA/
                  P16,GROUP OF 1 SENSILLA CAMPANIFORMIA/
                  P17,TIBIAL SENSILLA TRICHODEA/
                  P18,GROUP OF 2 SENSILLA CAMPANIFORMIA/
                  P19,GROUP OF 3 SENSILLA  CAMPANIFORMIA/
                  P20,PREAPICAL BRISTLE/
                  P21,APICAL BRISTLE/P22,NO. OF TIBIAL SPURS/
                  P23,NO. OF  TARSAL  BRISTLES/
                  P24,METATAPSAL 1 SENSILLA CAMPANIFORMIA/
                  P25,METATAPSAL 2 SENSILLA CAMPANIFORMIA/
                  P26,3RD SEGMENT 2 SENSILLA CAMPANIFORMIA/
                  P27,NO. OF UNGUES/
                  P28,NO. OF EMPODI/
                  P29,NO. OF PULVILLI/
                  P30,NO. OF UNGUITRACTOR PLATES/
                  P31,NO. OF APODEMES/
                  P32,COLUMN 1 OF S27/P33,COLUMN 2 OF ST7/
                  P34,COLUMN 1 OF S76/P35,COLUMN 2 OF ST6
  MISSING VALUES  ALL (9)
  ASSIGN MISSING  P32 TO P35,S32 TO S35 (9)
  READ INPUT DATA
  WRITE CASES     (F6.0,1X,2F3.0,1X,F2.0,1X,F1.0,1X,78F1.0)
                  ID,EXPT,HOUR,ECLSN,PS,P1 TO P31,P32,P33,P34,P35,
                  S1 TO S31,S32,S33,S34,S35
  FINISH
```

4. Recoding program run on recoded data

```
      DIMENSION IP(35),IS(35),IRC(35),IM(5)
    5 READ(5,100,END=99) IM,IP,IS
  100 FORMAT(I6,1X,2I3,1X,I2,1X,I1,1X,70I1)
      DO 10 I=1,35
      ISUM=IP(I)+IS(I)
      IPROD=IP(I)*IS(I)
      IRC(I)=0
      IF (ISUM .LT. 1) IRC(I)=1
      IF (ISUM .EQ. 1) IRC(I)=2
      IF ((IPROD .EQ. 0) .AND. (ISUM .EQ. 2)) IRC(I)=3
      IF ((IPROD .EQ. 1) .OR. (IPROD .EQ. 2)) IRC(I)=5
      IF (ISUM .EQ. 4) IRC(I)=6
      IF ((ISUM .GT. 4) .AND. (ISUM .LT. 9)) IRC(I)=7
   10 CONTINUE
      WRITE(6,110) IM,IRC
  110 FORMAT(I6,1X,2I3,1X,I2,1X,I1,1X,35I1)
      GO TO 5
   99 STOP
      END
```

5. Organization of recoded DFP leg data[1]

Columns	Data
1-17	same as on raw data card
19	1 = DFP leg
21	ST
22	SP
23	ThB
24	St4
25	St8
26	BH^-
27	St7
28	St6
29	St5
30	St3
31	St1
32	EB
33	Sc^+5
34	Sc^-8
35	Sc11
36	Sc1
37	TST
38	TSc2
39	TSc3
40	PAB
41	AB
42	Tsp
43	Tarsal Bristles
44	mSc1
45	mSc2
46	3Sc2
47	Un
48	Em
49	P
50	Unp
51	Ap
52	1St7[2]

APPENDIX B No. 5 (cont'd)

53	2st7
54	1st6
55	2st6

1. Recoded according to scheme described in Text

2. Added to the data set after it was entered into the computer.

6. Examples of the recoded data

```
45492 285 90  1 1 16555666566353535353355333365636666
45582 285 90  1 1 16555655533335535363365633366636563
45612 285 90  1 1 16567653333333333333333333333336533
45632 285 90  2 1 13515166533333566363365333333336666
```

7. Nearest neighbor analysis program run on recoded data

```
PRIMARY CHARACTER (PRI) MUST BE A DUPLICATION
SECONDARY CHARACTER (SEC) MUST BE A FULL DEFICIENCY
I/0 5=READ REQUEST; 6=READ DATA BASE; 7=OUTPUT
      INTEGER PRI,SEC,TER,CHAR(893,33),FREQ(33,33),ROW(33),
     A  SEL(33,12),VAR(33)
      DATA FREQ/1089*0/,ROW/33*0/
      DATA VAR/'ST  ','SP  ','THB ','ST4 ','ST8 ','HIB ','ST5 ',
     A  'SC3 ','ST1 ','EB  ','SC5 ','SC8 ','SC11','SC1 ','TST ',
     A  'TSC2','TSC3','PAB ','AB  ','TSP ','TAB ','MSC1','MSC2',
     A  '3SC2','UN  ','EM  ','P   ','UNP ','AP  ','1ST7','2ST7',
     A  '1ST6','2ST6'/
      WRITE(7,200)
      READ(5,100) NS,NDATA,((SEL(I,J),J=1,12),I=1,NS)
      READ(6,110)((CHAR(I,J),J=1,33),I=1,NDATA)
      DO 50 LOOP=1,NDATA
      DO 40 K=1,NS
      PRI=SEL(K,2)
      IF (CHAR(LOOP,PRI).LT.5) GO TO 40
      NSEC=SEL(K,1)
      DO 10 I=1,NSEC
      SEC=SEL(K,I+2)
      IF (CHAR(LOOP,SEC).NE.1) GO TO 10
      IF (I.EQ.NSEC) GO TO 25
      L=I+1
      DO 20 J=L,NSEC
      TER=SEL(K,J+2)
      IF (CHAR(LOOP,TER).LT.3) GO TO 40
   20 CONTINUE
   25 FREQ(PRI,SEC)=FREQ(PRI,SEC)+1
      ROW(PRI)=ROW(PRI)+1
      GO TO 40
   10 CONTINUE
   40 CONTINUE
   50 CONTINUE
      DO 30 I=1,NS
      NSEC=SEL(I,1)
      PRI=SEL(I,2)
   30 WRITE(7,210) VAR(PRI),ROW(PRI),
     1 (FREQ(PRI,SEL(I,J+2)),VAR(SEL(I,J+2)),J=1,NSEC)
      STOP
  100 FORMAT (2I5/(12I2))
  110 FORMAT (19X,6I1,2X,27I1)
  200 FORMAT ('1','NEAREST-NEIGHBOR ANALYSIS OF DUPLICATED',
     1 ' PRIMARY CHARACTER(PRI) FOR EACH EXCLUSIVELY DEFICIENT',
     2 ' SECONDARY CHARACTER(SEC)'/'-DUPLICATION',5X,'TOTAL',5X,
     3 'DEFICIENCIES',/)
  210 FORMAT ('0',3X,A5,8X,I4,6X,6(I3,A5,5X),/,' ',26X,
     A 4(I3,A5,5X))
      END
```

The data from the non-*Minute* clonal analysis was stored in two parts, on two types of cards, one containing the clone data and the second data from the legs containing the clones.

8a. Organization of the data on clone cards

Columns	Data
1	experiment number
3-4	treatment class[1]
5-7	Leg identification number[2]
8	Leg type[3]
10-12	hour of irradiation[4]
14	clone type[5]
16-17	clone location[6]
18-19	clone restrictions[7]
21-24	number of marked anterior trichomes
26-29	number of marked posterior trichomes
31-33	number of *y f* anterior bristles
35-37	number of *y f* posterior bristles
39-41	number of *sn*^{3} anterior bristles
43-45	number of *sn*^{3} posterior birstles

8b. Organization of the data on leg cards

Columns	Data
1	experiment number
3-4	treatment class[1]
5-7	leg identification number[2]
8	leg type[3]
21-25	number of anterior trichomes in leg
27-31	number of posterior trichomes in leg
33-35	number of anterior bristles in leg
37-39	number of posterior bristles in leg

1. 01-08=29° treated and irradiated, 01=irradiated at 24 hours
   02=irradiated at 48 hours, etc.
   09=29° treated unirradiated, collected before the irradiated batches
   10=22° constant unirradiated batch
   11-18=22° constant and irradiated, 11=irradiated at 24 hours
   12=irradiated at 48 hours, etc.
   19=29° treated unirradiated, collected after the irradiated batches

2. numbered from 001 → highest, in each treatment class

3. 1=Original portion of DFP
   2=Duplicate portion of DFP
   3=29° treated control
   4=22° treated control

4. given as hours after the midpoint of the egg collection

5. 1=*mwh* sn^3, *y f* twin
   2=*y f* single
   3=*mwh* sn^3 single

6. 11=thorax only eg. 16=thorax through tarsus
   22=coxa only
   33=trochanter only
   44=femur only
   55=tibia only
   66=tarsus only

7. column 18
   1=anterior only
   2=posterior only
   3=anterior and posterior

   column 19
   1=O clone
   2=D clone
   3=OD clone
   4=29° treated control leg clone
   5=29° control leg clone

Examples of the clone data cards

3	10132	24	3	3632	750	160				
3	10342	24	1	2632			30	10	14	5
3	10402	24	1	4512	10		1		1	
3	10492	24	1	2412	580		11		16	
3	10712	24	1	1633	1900	20	12	19	14	2
3	10962	24	1	4622		620		10		45
3	11252	24	1	4522		160		1		
3	11522	24	1	1632	4080	250		10		
3	11802	24	2	1632			180	106		

Examples of the leg data cards

```
3  11802          4620  1400 184 116
3  12772          4100  1150 175 100
3  13042          4100  1050 155  56
3  13372          2050   600 125  50
3  13532          3900   950 190 120
3  13702          1250   350  82  35
3  14162          2100   800 146  95
3  14232          3450   800  86  52
3  14262          3600   750  90  50
3  2 322          3400   600 170 115
3  2 452          3820  1100 260 145
3  2 592          3100   950 110  55
3  2 642          2650   800  65  40
3  2 982          3300   700 105  56
3  21112          4100  1650 175 105
```

APPENDIX C Markers Absent and Number of Cases in Unique Deficiency Classes Found in DFPs

Number of Cases	Markers Deficient
303	ST
56	(none)
47	ST, 2St6, St4, BH^-
39	ST, 2St6, St4, BH^-, 1St6
38	ST, 2St6, St4, BH^-, 1St6, St5
31	ST, 2St6, St4, BH^-, St5
22	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, St5
22	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, St5, 2St7
20	ST, 2St6
18	ST, St4, BH^-
17	ST, St4
16	ST, 2St6, St4, BH^-, 1St6, Sc11, St5
15	ST, 2St6, St4, BH^-, 1St6, St8, St5, 1St6
14	ST, 2St6, St4, BH^-, 1St6, St8, St5, Sc3
12	ST, 2St6, 1St6
10	ST, 2St6, St4
10	ST, 2St6, St4, BH^-, 1St6, St8, St5, Sc3, 2St7
9	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, 1St6
9	ST, 2St6, 1St6, 2St7
8	ST, BH^-
7	ST, 2St6, BH^-
7	ST, 2St6, St4, BH^-, St5, Sc3
6	ST, 2St6, St4, BH^-, 1St6, 2St7
5	ST, 2St6, St4, 1St6
5	ST, 2St6, BH^-, 1St6
5	ST, St4, BH^-, St5
4	ST, 2St6, St4, BH^-, 1St6, Sc11
4	ST, 2St6, St4, BH^-, St5, Sc11
4	ST, 2St6, St4, BH^-, St5, Sc3, Sc11
4	ST, 2St6, St5
3	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, 2St7, St5, TST, TSc3
2	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, 2St7, St8, St5
2	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, 2St7, St5, 1St7
2	ST, 2St6, St4, BH^-, 1St6, Sc11
2	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, TSc3
2	ST, 2St6, St4, BH^-, 1St6, St8
2	ST, 2St6, St4, BH^-, 1St6, Sc3
2	ST, 2St6, St4, BH^-, 1St6, St8, 2St7
2	ST, 2St6, St4, BH^-, 1St6, St8, 2St7, 1St7
2	ST, 2St6, St4, St5
2	ST, 2St6, St5, Sc3

APPENDIX C continued

2	ST, 2St6, BH^-, St5, 1St6
2	2St6
1	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, 2St7, St8, 1St7, St5, TSc3, PAB, mSc2
1	ST, 2St6, St4, BH^-, 1St6, Sc11 Sc3, 2St7
1	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, 2St7, St5, TST, TSc3, AB
1	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3
1	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, St5, TST
1	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, St5, TSc3
1	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, St5, St8
1	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, St5, 3Sc2
1	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, St5, St8, TST,TSc3
1	St, 2St6, St4, BH^-, 1St6, Sc11, Sc3, St5, TST, TSc3, AB
1	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, St5, TST, TSc3, AB, Tsp
1	ST, 2St6, St4, BH^-, 1St6, Sc11, Sc3, St5, TST, TSc3, AB, mSc1
1	ST, 2St6, St4, BH^-, 1St6, Sc11, EB
1	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, AB
1	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, TST, AB
1	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, Sc1, 2St7
1	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, TSc2, 2St7
1	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, St8, 2St7
1	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, PAB, 2St7
1	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, AB, mSc2, 2St7
1	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, TST, TSc3, 2St7
1	ST, 2St6, St4, BH^-, 1St6, Sc11, St5, TSc2, PAB, AB, Tsp, mSc1, mSc2, 3Sc2, Un, Em, P, Unp, Ap, 2St7
1	ST, 2St6, St4, BH^-, 1St6, St8, St5
1	ST, 2St6, St4, BH^-, 1St6, St8, St5, EB
1	ST, 2St6, St4, BH^-, 1St6, St8, St5, Sc^-8, Sc3
1	ST, 2St6, St4, BH^-, 1St6, St8, St5, Sc3, Sc^-8
1	ST, 2St6, St4, BH^-, 1St6, St8, St5, 2St7, 1St7
1	ST, 2St6, St4, BH^-, 1St6, St8, St5, St1, EB, Sc^+5
1	ST, 2St6, St4, BH^-, 1St6, St8, St5, St1, Sc5, 2St7
1	ST, 2St6, St4, BH^-, 1St6, St8, ThB, Sc^-8, 2St7, 1St7
1	ST, 2St6, St4, BH^-, Sc3
1	ST, 2St6, St4, BH^-, Sc11
1	ST, 2St6, St4, BH^-, Sc1
1	ST, 2St6, St4, BH^-, Sc3, Sc11
1	ST, 2St6, St4, BH^-, St5, Sc3, Sc1
1	ST, 2St6, St4, BH^-, St5, Sc3, St1, EB, Sc5, Sc^-8, Sc11
1	ST, 2St6, St4, TSc2, mSc1
1	ST, 2St6, St4, St8, 1St6
1	ST, 2St6, St4, St5, 1St6
1	ST, 2St6, St4, Sc11, 1St6
1	ST, 2St6, St4, 1St6, 2St7

APPENDIX C continued

1	ST, 2St6, St4, Sc3, Sc11, 1St6
1	ST, 2St6, St4, 1St6, 2St7, 1St7
1	ST, 2St6, St4, St5, Sc3, Sc11, 1St6
1	ST, 2St6, St4, St5, Sc3, Sc11, 1St6, 2St7
1	ST, 2St6, St4, St5, Sc3, Sc11, TST, TSc3, AB, Tsp, 1St6
1	ST, 2St6, St8
1	ST, 2St6, BH^-, Sc11
1	ST, 2St6, BH^-, Sc1
1	ST, 2St6, BH^-, St5, 1St6
1	ST, 2St6, St5, 1St6, 2St7
1	ST, 2St6, EB, 1St6, 1St7
1	ST, 2St6, St8, 1St6, 2St7, 1St7
1	ST, 2St6, BH^-, St5, 1St6, 2St7
1	ST,2St6, BH^-, Sc11, 1St7, 1St6
1	ST, 2St6, 1St6, 2St7, 1St7
1	ST, 2St6, St5, Sc3, 1St6, 2St7
1	ST, 2St6, St5, EB, 1St6, 2St7
1	ST, 2St6, St5, Sc11, 1St6, 2St7
1	ST, 2St6, BH^-, 1St6, 2St7, 1St7
1	ST, 2St6, BH^-, St5, Sc3, Sc11, 1St6
1	ST, 2St6, BH^-, St5, Sc3, Sc11, Sc1, TST, 1St6, 2St7
1	ST, 2St6, St5, Sc3, Sc11, TST, Tsc3, 1St6, 2St7, 1St7
1	St4
1	ST, EB
1	ST, Sc11
1	ST, Sc1
1	ST, 3Sc2
1	ST, BH^-, EB
1	ST, St4, BH^-, Sc3
1	ST, mSc1

APPENDIX D The Frequencies of Spontaneous Clones Divided into Size Classes

Type of Clone (Number)	Percent in each Size Class[1] 1	2	3	4	5	6
22° control (19)	63.2	26.3	5.3	5.3	0	0
29° treated control (16)	50.0	25.0	12.5	6.3	6.3	0
Original (20)	45.0	30.0	15.0	15.0	0	0
Duplicate (13)	46.2	23.1	23.1	7.7	0	0

1. Size classes based on number of cell divisions following segregation needed to produce that size of clone
   1 = 0-2 4 = 6-8
   2 = 2-4 5 = 8-10
   3 = 4-6 6 = 10-